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# TOXIC STRESS-INDUCED ADAPTIVE CELLULAR AND BEHAVIORAL RESPONSES IN *C. ELEGANS*

**PhD thesis**

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# List of abbreviations

| | |
|---|---|
| AMPA | α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| AMPK | AMP-Activated Kinase |
| ANOVA | Analysis of variance |
| BA | benzaldehyde |
| ccBA | “concentratus” benzaldehyde, undiluted benzaldehyde |
| ccDA | “concentratus” diacetyl, undiluted diacetyl |
| CI | Chemotaxis Index |
| CR | Calorie Restriction |
| crh-1 | CREB homolog |
| cyp-35B | Cytochrome p450 family B |
| DA | diacetyl |
| daf | Abnormal Dauer Formation |
| eat | EATing: abnormal pharyngeal pumping |
| FMRFamide | H-Phe-Met-Arg-Phe-NH2 amide sequence |
| FOXO | Forkhead box O |
| GFP | Green fluorescent protein |
| glo | gut granule loss |
| glr-1 | Glutamate Receptor family (AMPA) |
| GPCR | G-protein Coupled Receptor |
| gsk-3 | Glycogen Synthase Kinase 3 |
| gst-4 | glutathione S-transferase 4 |
| hsf-1 | Heat shock factor 1 |
| hsp-90 | Heat shock protein 90 |
| HSR | Heat Shock Response |
| IGF-1 | Insulin-like Growth Factor 1 |
| ITAM | Intermediate-term Associative Memory |
| ITI | Intertrial Interval |
| jnk | Jun N-terminal Kinase family |

| | |
|---|---|
| kgb-1 | Kinase, GLH-Binding 1 |
| LI | Learning index |
| LRO | Lysosome-related Organelle |
| LTAM | Long-term Associative Memory |
| mTOR | Mechanistic Target of Rapamycin |
| NGM | Nematode Growth Medium |
| NMDA | N-methyl-D-aspartate receptor |
| nmr-1 | NMDA class glutamate Receptor |
| npr | Neuropeptide Receptor family |
| NR | Nile Red |
| Nrf-2 | Nuclear factor, erythroid 2 like 2 |
| ocr | Osm-9 and Capsaicin receptor-Related |
| odr | Odorant Response Abnormal |
| osm | Osmotic Avoidance Abnormal |
| PA14 | *Pseudomonas aeruginosa* PA14 strain |
| PC | preconditioned |
| PI3K | Phosphatidylinositol 3 Kinase |
| pmk-1 | p38 Map Kinase family |
| PQ | paraquat dichloride hydrate |
| RNAi | Gene silencing by RNA interference |
| ROS | Reactive Oxigen Species |
| SAPK | Stress-activated Protein Kinase family |
| sgk-1 | Serum- and Glucocorticoid-inducible Kinase 1 |
| skn-1 | skinhead-1 |
| ST | Spaced Training |
| STAM | Short-term Associative Memory |
| tax | Abnormal Chemotaxis |
| TGF-β | Transforming Growth Factor beta |
| tir-1 | TIR (Toll and Interleukin 1 Receptor) domain protein |
| TRPV | Transient receptor potential vanilloid |

| | |
|---|---|
| $UPR^{ER}$ | Endoplasmic Reticulum Unfolded Protein Response |
| $UPR^{MITO}$ | Mitochondrial Unfolded Protein Response |
| wdr-23 | WD Repeat protein 23 |
| zip-2 | bZIP transcription factor family member |

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# 1 Introduction

## 1.1 Environmental stresses and adaptive responses

Challenging environmental alterations, i.e. stresses require adequate protective responses in multicellular organisms (Kültz, 2005; Selye, 1973). The sensory system is responsible for orchestrating perception of environmental cues representing safety or danger as well as internal, physiological state in order to generate optimal adaptive response. Hence, the "fight-or-flight" response is a result of complex, complementary interplay between cellular and behavioral adaptations coordinated by neuroendocrine signals (McCarty, 2016). Decision making can also be modulated by associative memories of past experiences wich help predict similar scenarios and reactions, to shape organismal fitness. Avoidance can be evoked by association of prior stressful experiences, such as physiological toxicity with sensory cues representing important resources (Cosmides & Tooby, 2013; Ozawa & Johansen, 2018). Such maladaptive repulsive behavior is characteristic in phobias, eating disorders and anxiety disorders which are challenging problems of our modern society (Garcia, 2017; Pittig, Treanor, LeBeau, & Craske, 2018).

## 1.2 *Caenorhabditis elegans* nematode as a model for stress biology

The *Caenorhabditis elegans* soil nematode is a versatile model organism due to fully mapped genome, neuronal network of its 302 neurons as well as highly conserved cellular stress pathways (Ardiel & Rankin, 2010; Bargmann, 2006). Furthermore, its short generation time and isogenic populations provide great opportunity to genetic manipulations as well as to utilize experiments with large populations (Brenner, 1974). Unfavorable environmental conditions as well as toxins and natural pathogens of *C. elegans* cause physiological damage (Rodriguez, Basten Snoek, De Bono, & Kammenga, 2013).

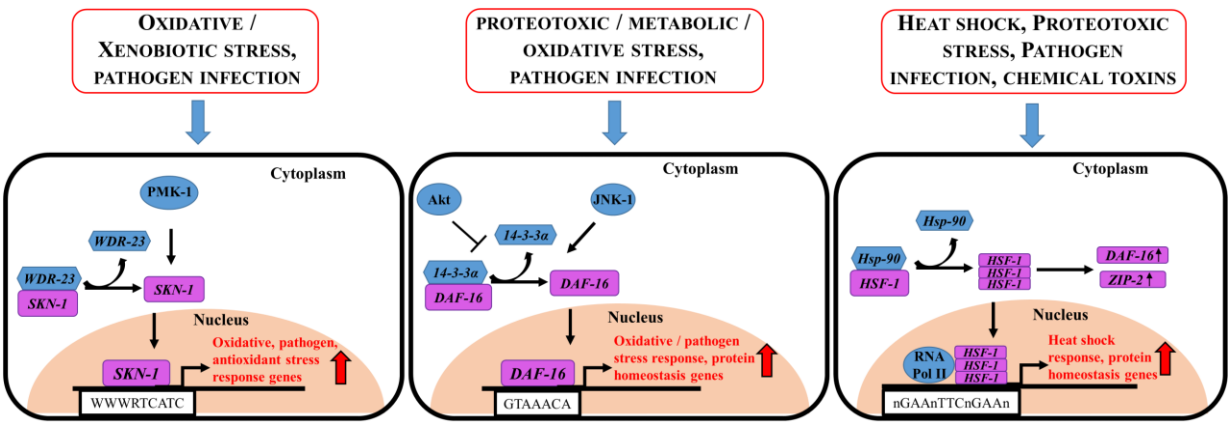

**Figure 1. Evolutionary conserved molecular stress pathways of *C. elegans*.** SKN-1, DAF-16 and HSF-1 stress transcription factors are activated upon different and overlapping environmental stimuli and release from inhibitory mechanisms (i. e. proteasomal degradation, inhibitory phosphorylation and chaperone binding, respectively) to stimulate different and overlapping stress transcriptional programs (Blackwell, Steinbaugh, Hourihan, Ewald, & Isik, 2015; Landis & Murphy, 2010; Ooi & Prahlad, 2017; Prahlad & Morimoto, 2009; V. Singh & Aballay, 2006).

To combat stresses, each cell possesses evolutionary conserved molecular surveillance mechanisms, such as the HSF-1 mediated heat shock response (HSR), the FOXO ortholog DAF-16 mediated oxidative-metabolic and Nrf-2 ortholog SKN-1 induced oxidative-xenobiotic stress response, as well as organellar stress responses such as the unfolded protein response of mitochondria ($UPR^{MITO}$) and endoplasmic reticulum ($UPR^{ER}$) (Blackwell et al., 2015; Sasaki & Yoshida, 2015; V. Singh & Aballay, 2006; Zečić & Braeckman, 2020). Master regulators of cellular stress, mainly transcription factors or upstream kinases are activated by several signal transduction pathways (Figure 1). The HSF-1 ortholog heat shock transcription factor is activated by trimerization. Chaperones such as Hsp-90 bind HSF-1 monomers to prevent the assembly of activated trimer structure. Upon stressful condition, Hsp-90 is titrated away by misfolded proteins to facilitate activation and DNA binding of HSF-1 trimers (Prahlad & Morimoto, 2009). Nuclear translocation of DAF-16 is constantly inhibited by AKT-1 mediated phosphorylation of the Insulin/IGF-1 signaling (IIS) pathway when ligand binds to the insulin receptor ortholog receptor DAF-2. Upon oxidative, proteotoxic and pathogen stress, DAF-16 is released from receptor-activated inhibition of 14-3-3 type proteins by phosphorylation-mediated allosteric changes, translocated to the nucleus to accelerate stress transcriptional program demanded by specific noxae (Murphy & Hu, 2013). Activation of SKN-1 is constantly inhibited by ubiquitination and degradation mediated by the Cul4-RING ortholog WDR-23 (WD Repeat protein 23) ubiquitin ligase (Blackwell et al., 2015). Interestingly, there are several activatory and inhibitory phosphorylation sites, for example GSK-3 (Glycogen Synthase Kinase 3), JNK-1 (Jun N-terminal Kinase), PMK-1 and MEK kinases of the RAS/MAPK pathway, AMPK (AMP-Activated Kinase), SGK-1 (Serum- and Glucocorticoid- inducible Kinase) and the two kinase complexes of mTOR (mechanistic target of rapamycin) to promote or prevent nuclear translocation and DNA binding (Landis & Murphy, 2010). Cellular stress pathways of *C. elegans* exhibit both modular and crosstalk responses to heat, oxidative, osmotic, and metabolic challenges, alteration in oxygen concentration, dietary restriction as well as pathogen invasion (Table 1).

Innate immune defenses are upregulated by toxins and secondary metabolites of pathogens *via* (i) the JNK-like MAP kinase cascade and p38 ortholog PMK-1 influencing (ii) the cAMP-dependent ATF-7 transcription factor, (iii) the *zip-2* bZIP transcription factor activated by toxin mediated inhibition of intestinal mRNA translation along with (iv) DAF-16, HSF-1 and SKN-1 induced antimicrobial protection and related antioxidant response to eliminate ROS generated by microbial colonization (Blackwell et al., 2015; Cohen & Troemel, 2015; Fletcher, Tillman, Butty, Levine, & Kim, 2019; Garsin et al., 2003; Kim et al., 2002; Papp, Csermely, & Sőti, 2012; V. Singh & Aballay, 2006). Induction of downstream transcription factors by nuclear translocation in *C. elegans* are easy to monitor *in vivo* due to its transparency. Elimination and neutralization of harmful agents, toxins, pathogens to maintain core cellular processes (i. e. gene expression, synthesis and degradation of primary and secondary metabolites, organelle-specific processes, and bioenergetics reactions), are priorities of cellular immune and stress pathways. In stress biology, the process of "hormesis" is the elevation physiological stress tolerance (i.e. increased survival to a subsequent lethal stress) by mild stresses *via* the efficient activation of cellular adaptive "fight" stress responses. In contrast, inadequate adaptive cellular responses lead to the phenomenon of "distress", a deficient capacity of the organism to combat the stress (Calabrese et al., 2007).

**Table 1. Evolutionarily conserved transcriptional stress pathways of *C. elegans*** (Blackwell et al., 2015; Brunquell, Morris, Lu, Cheng, & Westerheide, 2016; Ewbank, 2006; Murphy & Hu, 2013)**.**

| Master regulator | Human ortholog | Inhibitory protein/pathway | Cross-talk mediators | Main stresses |
|---|---|---|---|---|
| HSF-1 | Heat Shock Factor 1 | Hsp-90 | SIR-2.1 | Heat shock response |
| DAF-16 | Forkhead box O (FOXO) 1, 3, 4 | Insulin/IGF-1 (IIS) pathway | HSF-1, JNK-1, KGB-1, SKN-1, ZIP-2, GSK-3, SGK-1, TORC1, AAK-1 (AMPK) | Heat, immune, xenobiotic, osmotic stress, dietary restriction |
| SKN-1 | Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) | WDR-23 (Cul-4 type ubiqitin ligase) | DAF-16, JNK-1, PMK-1, SGK-1, TORC2 | Immune, electrophilic, xenobiotic, osmotic stress, dietary restriction |
| PMK-1/ KGB-1 | p38 Mitogen Activated Protein Kinase family 11 and 14 Mitogen Activated Kinase 8, 9 ,10 | VHP-1 (Dual specificity phosphatase) | SKN-1, MEK-1, ATF-7 | Immune and xenobiotic stress |
| ZIP-2/ CEBP-2 | ? CCAAT enhancer binding protein gamma (CEBPγ) | Proper ribosome assembly, mitochondrial and histone function | ATFS-1, ? | Immune responses |

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## 1.3 Chemosensation, learning and memory paradigms

Nematodes possess one of the simplest invertebrate nervous system with fully identified, invariant neuronal connectome with known size, position, electrical properties of neurons as well as basic synaptic connections. Each single neuron of *C. elegans* can specifically be ablated by laser microbeam, in order to gain "loss-of-neuron" phenotypes. Laser ablation screens helped scientists to identify distinct functions of neurons (Ardiel & Rankin, 2010; Bargmann, 2006; Hart & Chao, 2009).

Sensory signals, including olfactory and gustatory cues, are perceived by distinct chemosensory head neurons of the eleven bilaterally symmetric, abundantly ciliated amphid group. For example, pH and soluble attractants are sensed by ASE neurons, volatile attractants by AWA and AWC neurons, whereas AWB, ADL and ASH neurons detect repulsive odors. What might be the background of this highly sophisticated discrimination of sensory perception elicited by chemosensory neurons? According to laser ablation studies, the odorant diacetyl is sensed by the AWA pair of neurons to drive attractive response, whereas AWB neuron pair is responsible for avoidance in response to the odor 2-nonanone. In series of genetic screens, the G-protein coupled receptor (GPCR) ODR-10 were identified playing exclusive role of diacetyl perception, particularly expressed in AWA neurons. Animals carrying mutation in the *odr-10* gene are not attracted to diacetyl, which can be rescued by AWA-specific expression of ODR-10 in mutants. However, when ODR-10 is expressed specifically in AWB neurons, transgenic animals exhibited avoidance towards diacetyl odor source. Similarly, animals mutated in the *odr-1* gene failed to develop attraction towards benzaldehyde and isoamyl-alcohol, which phenotype can be rescued by AWC-specific overexpression of ODR-1 (Bargmann, 2006; Hart & Chao, 2009). These findings show that in the simple nervous system of worms, specific GPCR-s recognize the cue, whereas single neurons encode specific responses.

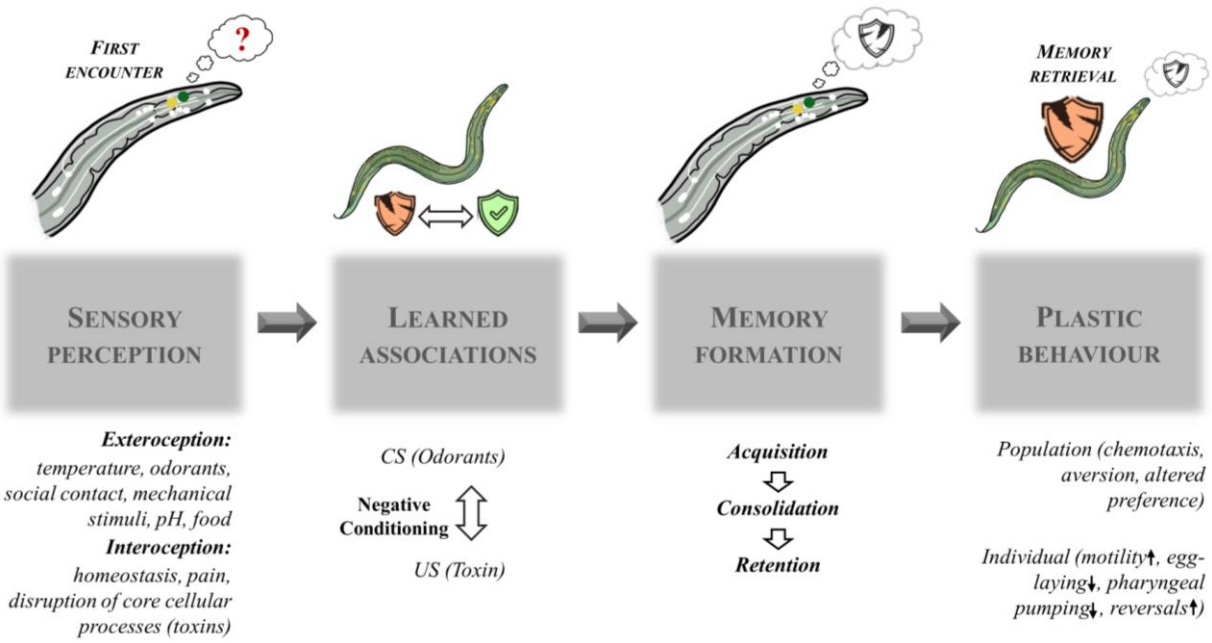


**Figure 2. Plasticity of *C. elegans* behavior by past experiences.** Environmental cues are processed by sensory integration of chemo, - thermo, - osmo, - mechanosensory and gustatory neurons as well as surveillance of internal homeostasis by interoceptors. Upon encountering harmful stimuli such as toxins or pathogens, the internal experience of toxicity (unconditioned stimulus, US) can be conditioned to the co-occurring environmental stimuli such as chemosensory cues (conditioned stimuli, CS) by associative learning. Strong, physiologically relevant new associations to cope with can be stored by the process of memory formation. Later, upon encountering only the conditioned cue of the past toxicity can evoke learned behaviors by memory retrieval.

*C. elegans* offers a wide repository of behavioral patterns elicited by social contact, pheromones, as well as environmental cues of nutrition and danger. Sensation of noxious stimuli can interrupt continuous sinusoid movement and evoke aversive behavioral patterns of simple reversal, “omega bend” shaped turn-off by head of the animal bends towards its tail, or a complex behavioral pattern of reversal-to-omega turn called “pirouette” behaviors (CROLL, 2009).

*C. elegans* exhibits behavioral plasticity based on prior experiences, indicating efficient learning and memory processes (Fig. 2, *24*). Habituation is a form of non-associative learning in which an innate (non-reinforced) response to a stimulus decreases after repeated or prolonged presentations of that stimulus. For example, organisms may habituate to repeated mechanical stimuli or prolonged odor exposure when they learn these have no biological consequences. Dishabituation is the reestablishment of the decremented response by latency or a different stimulus. Non-associative sensitization is the amplification of the response by repeated harmful stimuli, whereas desensitization – in *C. elegans* often called chemosensory

adaptation – occurs when repeated positive or negative stimuli become redundant, due to chemosensory receptor downregulation/internalization. Intriguingly, re-appearance of the environmental cue alone, or dysfunctions in glutamate neurotransmission usually disrupts habituated or sensitized plastic behavior (Sasakura & Mori, 2013). Investigating the tap withdrawal response (i.e. the behavioral backward locomotory response to gentle mechanical stimulus), scientists were also able to distinguish short-term and long-term memory of habituation as CRH-1 [homologous to cyclic AMP-response element-binding protein (CREB)] is required for long-term, but not short-term memory of habituation. Interestingly, both short and long-term habituation requires the non-N-methyl-D-aspartate (NMDA) glutamate receptor subunit GLR-1 as well as the glutamate vesicular transporter EAT-4, homologs of which mediate the long-term potentiation, a molecular basis of learning in various animals (Lau, Timbers, Mahmoud, & Rankin, 2013; Sasakura & Mori, 2013).

When nematodes are trained to link a conditioned stimulus (CS), such as a sensory cue with an unconditioned stimulus (US), to which an instinctual response exist, as the paradigm of both positive or negative "classical conditioning", the evolutionary conserved phenomenon of associative learning or context conditioning takes place (*31*, *33*, *34*, Fig. 3). Conventionally, *C. elegans* can be trained to associate the presence or absence of food or toxins (US) with simultaneously presented gustatory or olfactory cues as well as temperature (CS) to alter behavior. In mutagenesis screens, the respective mutants that exhibit compromised attractive or aversive associations are classified as *lrn* mutant strains as well as *glr-1* mutants. These discoveries indicate the role of glutamatergic neurotransmission in both non-associative and associative forms of learning (Lau et al., 2013).

Formation of associative memories enables long-term behavioral plasticity (*4*, *31*, Fig. 2). During conditioning, "memory acquisition" stores association between CS and US, "memory retention" maintains storage, while "memory retrieval" invokes the US-elicited plastic behavior upon reoccurrence of CS. Nematodes possess short-term, intermediate-term and long-term associative memories (STAM, ITAM, LTAM) according to the time span of memory retention (Amano & Maruyama, 2011; Ardiel & Rankin, 2010). Traditionally, metazoans form distinct associative memories by several conditioning techniques (Hawkins, Carew, & Kandel, 1986; Jones, 1962; Kandel, 2001). In *C. elegans*, a simple simultaneous exposure of CS and US initiates associative learning but newly acquired plastic behavior can be retrievable only in the short-term. Repeated exposure to stimuli without intervals called "massed training" is able to form STAM and ITAM with memories stored no more than 2 hours. Repeated training sessions using intertrial intervals (ITI) called "spaced training" generates the formation of long-

term memories (2 to 24 hours) which require new protein synthesis and is shown to be regulated by *CRH-1* (Amano & Maruyama, 2011; Smolen, Zhang, & Byrne, 2016). Consequently, both translation inhibition by cycloheximide and mutation in *crh-1* disrupt spaced training evoked LTAM but don't affect massed training induced STAM or ITAM (Stein & Murphy, 2014). In contrast, *glr-1* is required only for short-term storage of associations, whereas both short, - and long-term odor context conditioning were disrupted in the absence of the *C. elegans* homolog of the NMDA NR1 glutamate receptor subunit, *nmr-1* (Lau et al., 2013). Interestingly, the conserved Insulin/IGF-1 signaling (IIS) pathway regulates acquisition of positive associative memories as well as starvation-induced aversive associative memories (Lin et al., 2010; Stein & Murphy, 2014).

A specific, conserved form of lasting memory acquired in a critical perinatal period is sensory imprinting. Imprinted behaviors is critical for an animal's survival or reproductive success (fitness). In *C. elegans*, it is acquired in the L1 larval stage and is retrieved in adult stage. It is associated with preference of favorable environmental conditions shaped by the perinatal (imprinted) experiences. Imprinting probably helps to make bonding to the cues of safety, such as those signaling food (Remy & Hobert, 2005). However, *C. elegans* is also capable of storing the sensory cues of pathogenic *Pseudomonas aeruginosa* bacteria when encounters it during L1 stage to enable more efficient aversion later in adulthood. Retrieval of this long-lasting associative memory also generates repulsive behavior when the toxic agent exotoxin A (ToxA) of *P. aeruginosa* is presented (Jin, Pokala, & Bargmann, 2016).

Not only behavioral, but cytoprotective responses can also form acquired memories by association of odor (CS) and starvation (US) and successfully retrieved by the olfactory cue presented alone (Eliezer et al., 2019). The abovementioned finding together with the discovery of lifelong memories induced by pathogenesis of L1 larvae (Gecse, Gilányi, Csaba, Hajdú, & Sőti, 2019) provided the intriguing idea that early life hormetic stress and activation of cytoprotective responses might contribute to adulthood's stress resistance by retrieving imprinted physiological defense memories.

## 1.4 Interactions between neurobehavioral and non-neuronal intracellular stress defenses

The nervous system oversees adaptive processes via neuroendocrine signaling by (i) neuropeptides, (ii) TGF-ß/DAF-7 and Insulin-like ligands along with (iii) neurotransmitters, representing a comprehensive modulation of longevity, gut immune defenses, organellar stress responses and fat metabolism resulting in optimal behavioral outcome (Schild & Glauser, 2013;

J. Singh & Aballay, 2020; Stein & Murphy, 2012). Behavior is an immediate response, while the transcriptional and mainly translational upregulation of cytoprotective responses of molecular stress pathways requires more time (hours or days). These cell-nonautonomous regulations influence DAF-16/FOXO and Nrf-2/SKN-1 regulated stress responses, HSF-1 activated HSR as well as JNK-like MAP kinases regulated immune- and detoxification responses in non-neuronal cells (Foster et al., 2020; Ogg et al., 1997; Ooi & Prahlad, 2017; Park, Tedesco, & Johnson, 2009; Pukkila-Worley, 2016).

There is emerging evidence that autonomous machinery of cellular adaptive responses possess impact on neuronal integration. RNA interference based silencing – which is limited to non-neuronal cells – of core cellular processes elicited induction of cytoprotective and immune responses as well as aversive behavior (Melo & Ruvkun, 2012; Pukkila-Worley, 2016). *Ruvkun et al.* demonstrated the role of Jnk-like MAP kinase pathways in RNAi-induced food avoidance as well as serotonergic signaling dependent associative learning for future feeding decisions (Melo & Ruvkun, 2012; Shore & Ruvkun, 2013). Aversive behavior is prominently delayed in the course of the Gram-negative *Pseudomonas aeruginosa* infection. In this particular case, animals show an initial preference of the pathogenic bacteria against nutritional *E. coli* strains, while aversion begins only hours later by intestinal colonization of pathogens and the production of toxic compounds, including ROS (Meisel & Kim, 2014). Delayed aversion is potentially the result of the overwhelmed detoxification of enterocytes, showing a clear influence of non-neuronal cells on behavioral outcome (Horspool & Chang, 2017). Additionally, sensory integration of infection-induced intestinal bloating is orchestrated by neuropeptidergic signaling as well as serotonergic signaling (J. Singh & Aballay, 2019, 2020).

Both non-neuronal-restricted gene silencing experiments and the phenomenon of *P. aeruginosa* elicited aversion suggest an adaptive interplay between cytoprotection of non-neuronal cells and behavioral response orchestrated by neuronal integration of signaling from both tissue damage and chemosensation. Nonetheless, a clear molecular evidence of such connection in decision making as well as in associative learning is missing.

## 1.5 Benzaldehyde and diacetyl

Several studies reported toxicity of both benzaldehyde and diacetyl in cell cultures as well as in vivo mammalian animal models (Andersen, 2006; Brass & Palmer, 2017b). The pleasant odor utilized by food and perfume industry is also well-known (Clark & Winter, 2015; Wen et al., 2014). Odorant compounds of several food bacteria were identified and

demonstrated to be sensed by AWC and AWA pair of neurons of nematodes. Both benzaldehyde and diacetyl are food bacteria derived odors, which elicit attractive behavior in low concentrations. Benzaldehyde perceived through GPCR receptor binding of AWC neurons via the *ODR-3* G-protein, *ODR-1* guanylyl-cyclase and cGMP-gated *TAX2/TAX4* cation channel; whereas diacetyl sensed by the ODR-10 receptor of AWA neurons, processed via *ODR-3* mediated poly-unsaturated fatty acid synthesis and activation of *OCR-2/OSM9* TRPV channel (Hart & Chao, 2009). Disruption in any element of these pathways abrogates the perception and attractive response to benzaldehyde and diacetyl, moreover in case of *odr-3* mutation, neither odors are perceived (Figure 3). However, attraction is mitigated and aversion develops at higher concentrations (Nuttley, Harbinder, & van der Kooy, 2001; Yoshida et al., 2012) which phenomenon was, perhaps erroneously described as habituation since high doses of both BA and DA are toxic (Andersen, 2006; Brass & Palmer, 2017a). In *C. elegans,* ASH chemosensory neuron was identified perceiving and forwarding polymodal cues of both volatile or soluble repellents, osmotic or heavy metal stresses via *ODR-3* and *OCR-2/OSM9* mediated GPCR-signaling.

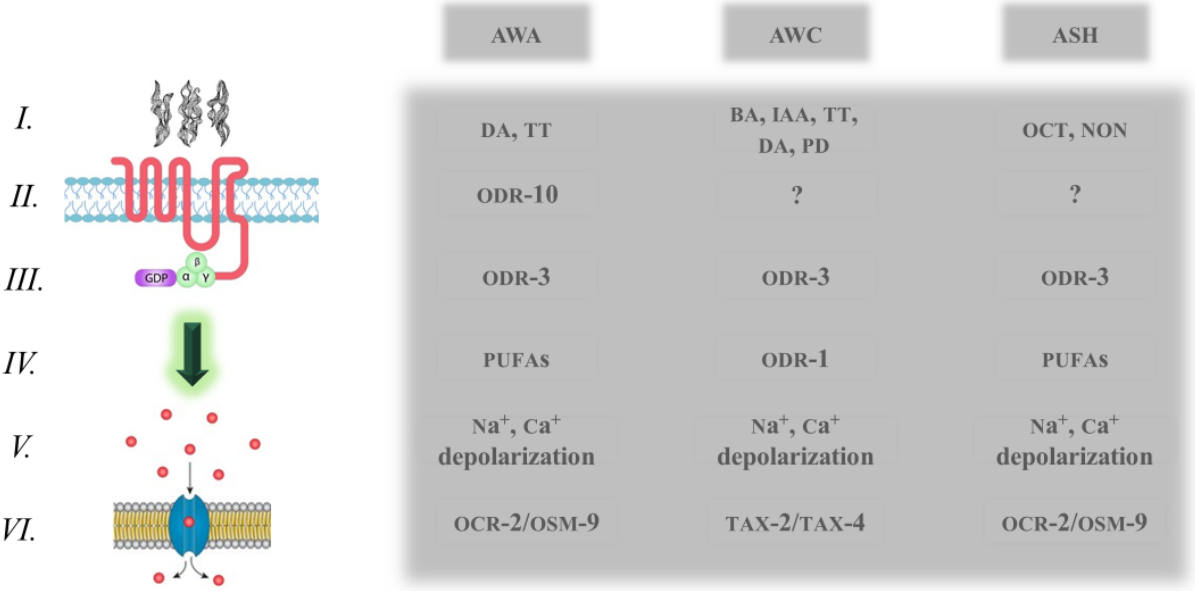


**Figure 3. Schematic signal transduction and main contributors of olfaction in AWA, AWC and ASH chemosensory neurons.** (I) Odorant molecules elicit a sophisticated activation of specific GPCR-s in ciliated amphid neuron membranes. (II) The density of a particular GPCR is chemosensory neuron specific, for example ODR-10 receptor is characteristic to AWA neurons. (III) GPCR-activation induces disassembly of intracellular heterotrimeric G-protein and the $G_i$-like Gα subunit product of *odr-3* gene is responsible for odor induced signal transduction. (IV) Poly-unsaturated fatty acids (PUFAs) in case of AWA and ASH, and membrane associated ODR-1 guanylyl-cyclase generated cGMP in case

of AWC emerge as second messengers (V) to enhance $Na^+$ and $Ca^{2+}$ depolarization by (VI) OCR-2/OSM-9 TRPV-channels and TAX-2/TAX-4 cGMP-gated ion channels, respectively.

During investigation of odor-induced behaviors, we and others observed that worms exhibited an initial attraction towards and delayed aversion of the odor source of undiluted 100% benzaldehyde – so called benzotaxis – which was also observed with undiluted diacetyl ((Nuttley et al., 2001); see also Figure S1 of our publication, (Hajdú, Gecse, Taisz, Móra, & Sőti, 2021)). Disruption of the TRPV-channel by *osm-9* mutation altered neither the attractive nor the aversive behavior. However, mutation in the ODR-3 Gα subunit abrogated the attractive phase whereas the aversive phase remained unchanged (Figure 4 taken from (Nuttley et al., 2001; Tsui & van der Kooy, 2008)). The exclusive preservation of benzaldehyde avoidance in the *odr-3* chemosensory mutant, and its sensitivity to dishabituation suggested that aversion is an independent behavior which appeared after habituation to the attractive stimulus in the absence of food. Delayed aversion of benzotaxis is highly similar to the well-studied behavioral response elicited by *P. aeruginosa* infection (Meisel & Kim, 2014).

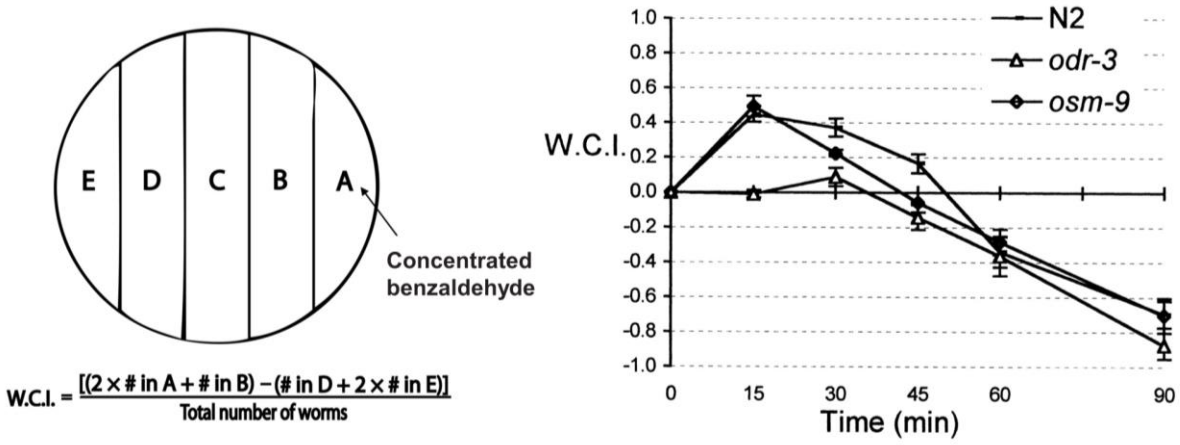


**Figure 4. Kinetic benzaldehyde chemotaxis (benzotaxis) curve of wild-type (N2), *odr-3* and *osm-9* mutants.** W.C.I. (Weighted Chemotaxis Index) of benzotaxis represents the behavioral index of populations on a chemotaxis plate divided to sectors, where the number of animals in each sector has a value according to a weighted formula (shown above). Positive W.C.I. value means attraction towards, whereas negative value demonstrates aversion of the odor source. Wild-type populations and *osm-9* mutants elicit an initial attraction towards and delayed aversion of concentrated benzaldehyde odor source. *odr-3* mutants show no attraction and maintain the aversion of benzaldehyde comparable to N2 (Nuttley et al., 2001; Tsui & van der Kooy, 2008).

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# 2 Objectives

The main objectives of my PhD studies were the investigation of expression, relationship and learning of cytoprotective and behavioral defense mechanisms in response to toxic stress. The specific aims were as follows:

1A. To establish and characterize a nematode model of volatile-induced toxic stress

1B. To investigate how intracellular stress responses affect stress-induced aversive behavior and learned behavioral decisions.

1C. To investigate whether toxic volatile stress in adulthood forms a cytoprotective memory in non-neuronal cells

2. To assess how the retrieval of an imprinted cytoprotective memory interferes with stress tolerance in adulthood.

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# 3 Results

The text of the two published articles is the most expedient presentation of the results, thus this chapter is based on the text of the results section of our articles entitled *Toxic stress-specific cytoprotective responses regulate learned behavioral decisions in C. elegans* (Hajdú et al., 2021) and *A cellular defense memory imprinted by early life toxic stress* (Gecse et al., 2019) with minor changes.

## 3.1 Distinct adaptive responses elicited by volatile-induced toxic stress

The discovery that unlike isoamyl-alcohol, food bacteria-secreted diacetyl and benzaldehyde odors are able to induce ODR-3 independent repulsion in concentrated form led us to the hypothesis of direct toxicity of these compounds besides the idea of concentration-dependent switch in chemosensation. To test this, we investigated physiological effects induced by undiluted ("concentratus") benzaldehyde (ccBA) and undiluted diacetyl (ccDA).

### 3.1.1 Behavioral aversion is triggered by undiluted volatile toxicity

We hypothesized that if aversion is a defensive behavioral response and is independent of habituation and/or olfactory adaptation (i. e. diminished behavioral by repeated or extended presentation of a single stimulus), then ccBA will also trigger nematodes to leave the food lawn rich in chemosensory and nutritive stimuli. To investigate this possibility, we placed a ccBA drop on a parafilm in the middle of a central E. coli OP50 lawn, where worms acclimatized for 30 min and monitored food avoidance (Fig 3a). Using a ccBA dose proportionally considering the plate volume used in kinetic chemotaxis experiments, we observed that while mock-exposed worms remained on the lawn after 50 min, the majority of the ccBA-exposed worms left the food (Fig. 5b). Diacetyl (DA), a chemically unrelated food odor, is also triggered an initial attraction followed by a delayed aversion (Hajdú et al., 2021; Nuttley et al., 2001). We found that both ccBA and ccDA elicited concentration-dependent food aversion phenotypes (Fig. 5c). Further, we observed a time-dependent development of food aversion for both volatiles (Fig. 5d, e), which, even though food was present, showed a faster kinetics, than that in the kinetic chemotaxis experiments. As starvation induces both adaptation and habituation, both neuronal mechanisms to the undiluted odors might occur in the absence of food. However, worms not only decreased their sensory perception of, or their interest towards, inconsequential odors but actively vacated the lawn to reach the furthest possible distance from the odor source. Taken together, giving up the advantage of nutrition is a consequence of a defensive behavioral decision to avoid a harmful stimulus.

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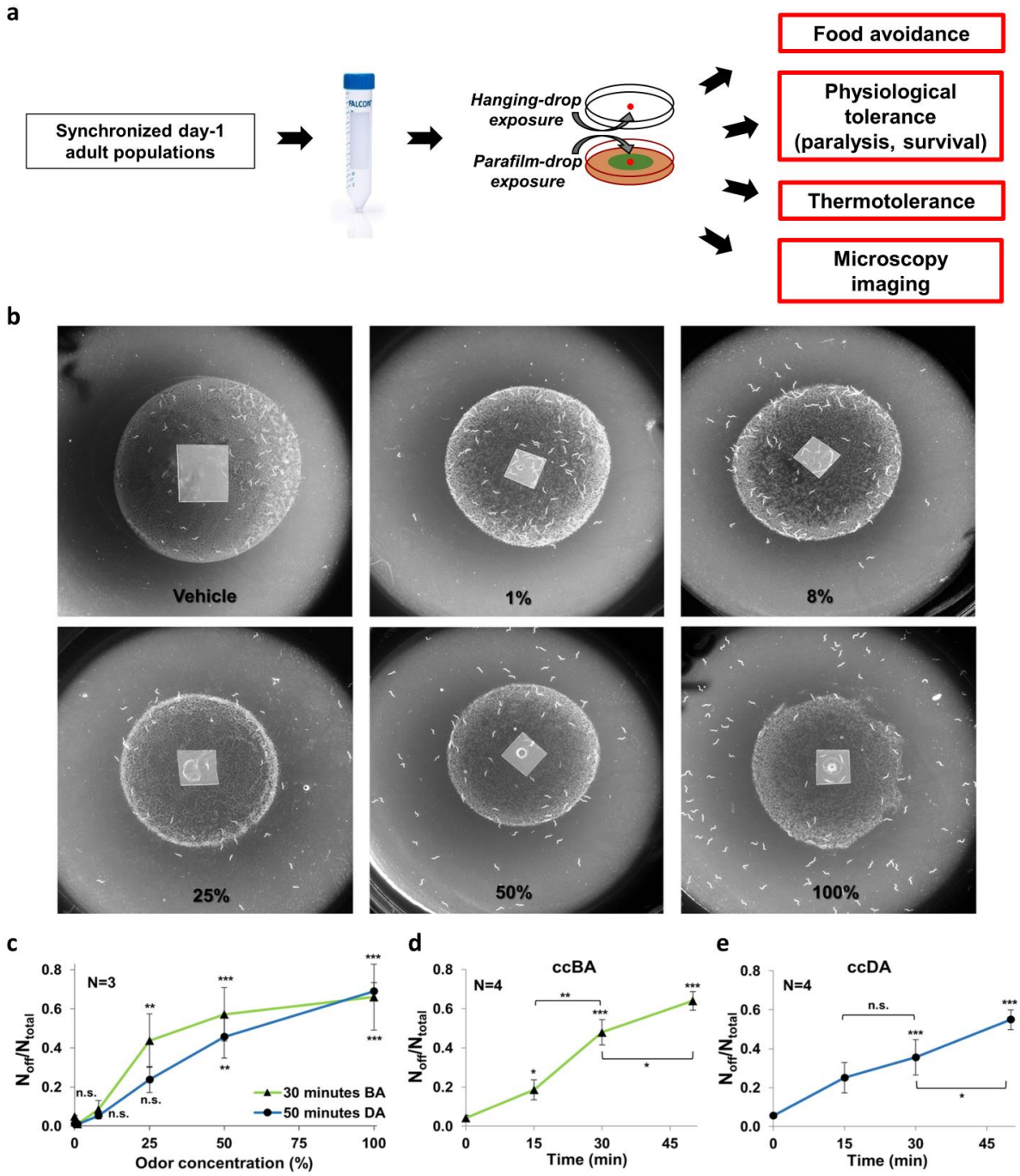


**Figure 5. Undiluted benzaldehyde (ccBA) and diacetyl (ccDA) induce food aversion** (a) Experimental setup for odor toxicity. After washing day-1 adult populations from nutritional NGM-plates in M9 buffer, a given number of animals were dropped onto survival or behavioral test plates. Concentrated BA or DA was placed in a total volume of 1 µl or 4 µl on the lid of the plates or in the middle of the bacterial lawn. (b) Representative images of food leaving behavior in response to a 50-min exposure to various concentrations of BA. BA was placed in an ethanol vehicle in a total volume of 1 µl in the middle of the bacterial lawn. c) Dose dependence of food avoidance after a 30-min exposure to BA or a 50-min exposure to DA. (d) Time dependence of 1 µl ccBA-induced food avoidance. (e) Time dependence of 4 µl ccDA-induced food avoidance. $N_{off}/N_{total}$ values calculated by

the $N_O$ of animals outside the bacterial food lawn / $N_O$ of total animals on the plate. Data are expressed as mean ± SEM. N, number of independent experiments. p values were obtained by one-way ANOVA with Fisher's LSD post hoc test. n.s., not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

To further address if animals avoided ccBA and ccDA because of toxic effects, we evaluated the paralysis rate of worms subjected to different undiluted odorant doses in our "hanging-drop" volatile toxicity model. We found that longer ccBA and ccDA hanging-drop exposures to higher doses induced extensive paralysis in a dose- and time-dependent manner (Fig. 6a, b). Then, we estimated toxicity by monitoring survival the day after hanging-drop exposure to the highest doses of the respective undiluted odors and observed that ccBA and ccDA similarly induced death in an exposure time-dependent manner (Fig. 6e, f). Accordingly, we detected a marked deterioration of the internal structure of animals after the exposure to the highest dose of ccDA, compared to a preserved morphology after that of ccBA (Fig. 6d). Importantly, extended exposure to doses of ccBA and ccDA used in food leaving assays was not apparently toxic *per se* (Fig. 6a, b), but both impaired thermotolerance (i.e., the ability to withstand heat stress) (Fig. 6c). The impaired stress tolerance, paralysis, and death by increasing doses of ccBA and ccDA represent a progressive disruption of physiological homeostasis. Based on these findings, we hypothesized that the behavioral avoidance of the undiluted odorants may be a consequence of their toxic effect demonstrated in our volatile toxicity model.

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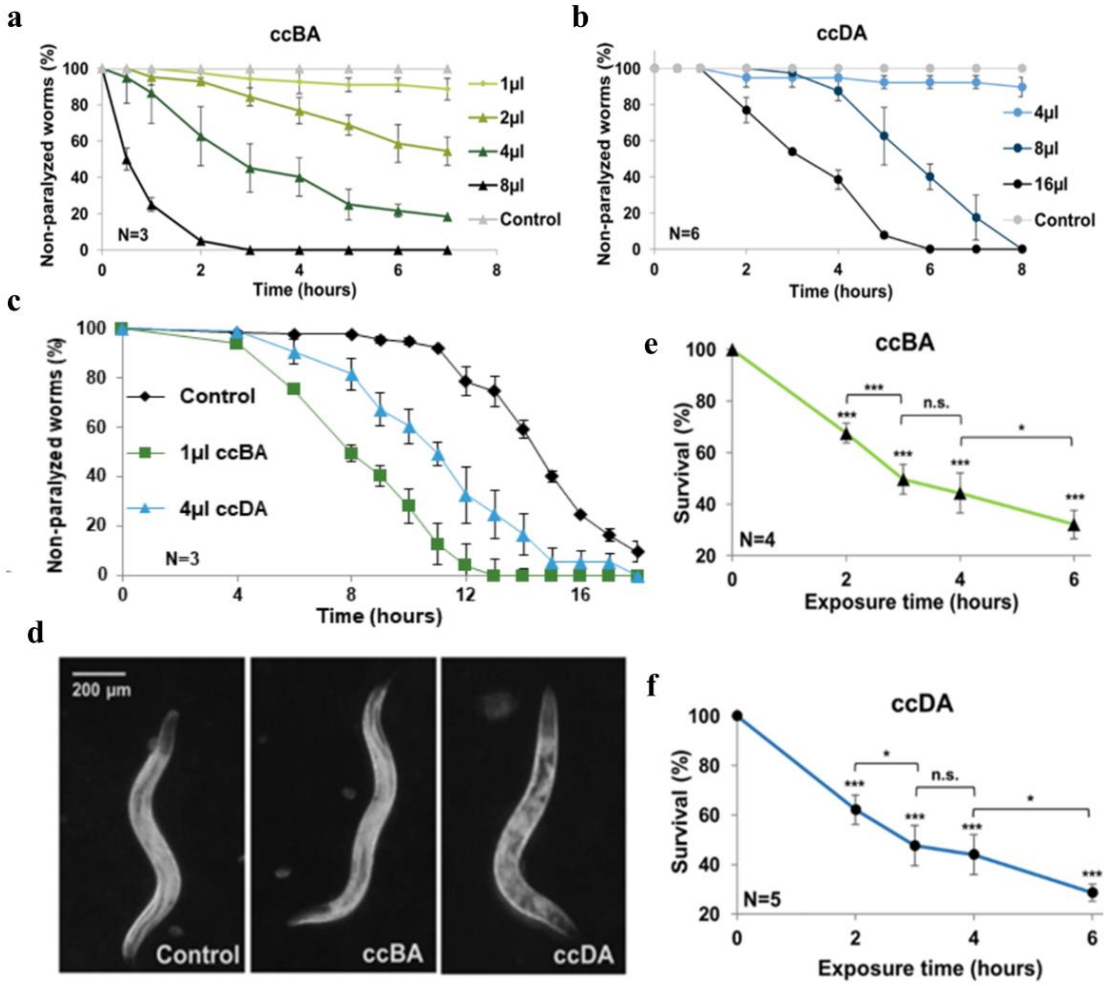


**Figure 6. Undiluted benzaldehyde (ccBA) and diacetyl (ccDA) induce toxicity.** (a) Time dependence curves of paralysis to various doses of ccBA. (b) Time dependence curves of paralysis to various doses of ccDA. (c) Continuous exposure to 1 µl ccBA and 4 µl ccDA impairs thermotolerance, the resistance to heat stress. (d) Representative stereomicroscopic images of worms 14 h after a 3-h exposure to 8 µl ccBA or 16 µl ccDA. (e) Exposure time dependence of survival to the highest, 8 µl dose of ccBA. (f) Exposure time dependence of survival to the highest, 16 µl dose of ccDA. Survival was scored 14 h after the end of exposures. Mean durations of heat shock that induced 50% paralysis by log rank (Mantel-Cox) test were as follows: 14.46 ± 0.23 hours for vehicle treated control, 10.74 ± 0.42 hours for ccBA-exposed (p=0.0001 compared to control), 12.45 ± 0.43 hours for ccDA-exposed (p=0.011 compared to control). The mean durations of odorant exposure that induced 50% paralysis by log rank (Mantel-Cox) test were as follows: ccBA - 2 µl 5.27 h ± 0.17 h, 4 µl 2.94 ± 0.21 h, and 8 µl 0.94 ± 0.14 h; ccDA - 8 µl 5.68 ± 0.20 h and 16 µl 3.46 ± 0.17 h. Compared to 1 µl BA or 4 µl DA treatments $p < 0.001$ in all conditions. Data are expressed as mean ± SEM. N, number of independent experiments. p values were obtained by one-

way ANOVA with Fisher's LSD post hoc test. n.s., not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

### 3.1.2 Odorant preconditioning evokes distinct modes of adaptation

We observed that transient exposure to higher doses of ccBA and ccDA increased motility (Fig. 7a), suggesting that perception of toxic stress increases locomotor activity which may help instantly escape from the threat. Interestingly, the increased motility returned to baseline after removing ccBA but showed a sustained elevation after the removal of ccDA (Fig. 7a and (Hajdú et al., 2021). Moreover, we found that after an extended 2h exposure to ccBA, animals started to return to the bacterial lawn, whereas the same exposure to ccDA further increased aversion (Fig. 7b). Thus, the adverse physiological effects of ccBA might be eliminated faster than those of ccDA. We reasoned that a preconditioning exposure might differentially affect the defensive behavior to ccBA and to ccDA. To test this, we preconditioned the worms by exposing them to the same doses of odorants for 4 h on a large bacterial food lawn in order to prevent food avoidance during preconditioning. After washing, we placed them on a fresh, small bacterial lawn containing the same odorant doses and monitored their lawn avoidance behavior (Fig. 7c). We found that preconditioning with ccBA (BA PC) largely diminished ccBA-induced aversion for the entire duration of the experiment (Fig. 7d). In contrast, preconditioning with ccDA (DA PC) robustly increased the speed of ccDA lawn avoidance, reaching almost maximal value within 15 min (Fig. 7e). Thus, the fast ccDA lawn avoidance, despite the reduced motility (Hajdú et al., 2021), appears to be the consequence of a directed navigation away from a familiar noxious stimulus. Preconditioning-induced behavioral changes were apparent at 2 h and were most pronounced at 4 h of pre-exposure (Fig. 7f, g).

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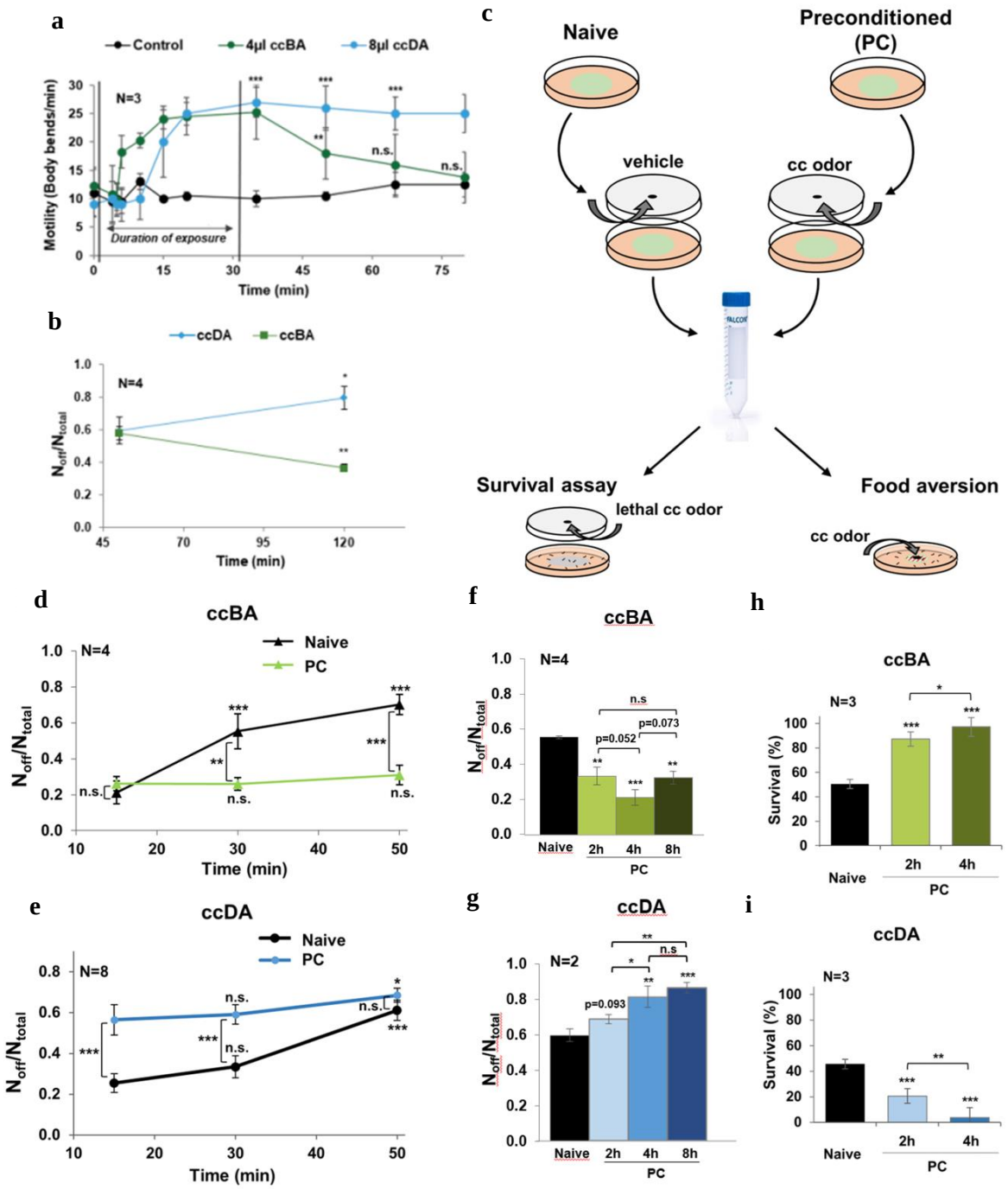


**Figure 7. ccBA preconditioning (BA PC) induces behavioral and physiological stress tolerance, while ccDA preconditioning (DA PC) induces sensitization.** (a) Motility assays show reversible vs. sustained elevation in locomotion in response to a 30-minute exposure to ccBA vs. ccDA. (b) Food aversion data showing that extended odor exposure to ccBA decreases, whereas that to ccDA further increases aversive behavior. (c) Experimental setup for preconditioning, followed by food aversion and survival tests. Animals were exposed to a hanging drop of undiluted odor (preconditioned, PC) or vehicle (naive), washed, and assayed for food avoidance or survival by exposure to the same or a lethal

odor dose, respectively. (d) Food aversion induced by 1 µl ccBA of naive and ccBA-preconditioned (BA PC, 1 µl for 4 h) animals at different time points. (e) Food aversion induced by 4 µl of ccDA of naive and ccDA-preconditioned (DA PC, 4 µl for 4 h) animals at different time points. (f) ccBA-induced food avoidance as a function of duration of preconditioning exposure. (g) ccDA induced food avoidance as a function of duration of preconditioning exposure. (h) Survival of naive and ccBA-preconditioned worms 14 h after a 3-h exposure to 8 µl ccBA. (i) Survival of naive and ccDA-preconditioned worms 14 h after a 3-h exposure to 16 µl ccDA. Data are expressed as mean ± SEM. N, number of independent experiments. p values were obtained by one-way ANOVA with Fisher's LSD post hoc test. n.s., not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

For the increased capacity of worms to remain in the presence of toxic ccBA, we used the term "behavioral tolerance," to the analogy of physiological stress tolerance (i.e., the capacity to resist physical stress by engaging physiological defenses, such as cytoprotective stress responses). To investigate whether the contrasting behavioral responses evoked by the two volatiles were accompanied by similar outcomes in physiological stress tolerance, we preconditioned the worms with the lower, non-toxic odor doses used in the food leaving assays, then subjected them to lethal odor doses for 3 h and evaluated their survival 14 h after the end of the exposure. With increasing preconditioning time, we observed a robust survival increase on ccBA and a complete survival decline on ccDA (Fig. 7h, i), representing a protective (hormetic) effect of ccBA and a debilitating (distressing) effect of ccDA preconditioning. Hormesis and distress are well-known phenomena in stress biology and suggest efficient or insufficient physiological responses to the stress induced by ccBA or ccDA exposures, respectively (Calabrese et al., 2007). Thus, ccBA preconditioning induces behavioral and physiological stress tolerance, while ccDA preconditioning induces behavioral sensitization and physiological distress. These results suggest that nematodes can mount efficient physiological protection against ccBA but can only engage more alert behavioral defense through sensitization against ccDA.

## 3.2 Adaptive cellular responses induced by odor preconditioning

Next, we asked if the efficient *vs.* insufficient physiological protection against ccBA and ccDA exposure might be reflected in the differential mobilization of cellular defense responses to the respective toxic stresses. In agreement with our findings on the toxicity of ccBA, previous studies demonstrated that BA induced oxidative stress *(52)*. Therefore, we tested various oxidative stress response pathways that might be involved in the physiological adaptation to ccBA.

### 3.2.1 Activation of stress regulators DAF-16/FOXO and SKN-1/Nrf-2 and their target genes upon BA PC

Key transcriptional regulators of *C. elegans* stress pathways such as DAF-16/FOXO and SKN-1/Nrf-2 is easy to monitor by visible nuclear translocation using fluorescence-tagged transgene animals. Using the TJ356 strain expressing GFP-tagged DAF-16, we observed that the same ccBA dose used for preconditioning induced a strong nuclear translocation of DAF-16 after 30 min, comparable to that induced by heat stress. However, DAF-16 remained cytosolic in response to ccDA (Fig. 8a).

Translocation of the oxidative-xenobiotic stress master regulator SKN-1::GFP in the LD001 strain was induced by a 30-min exposure to ccBA, comparable to that seen upon treatment with the oxidative agent paraquat (PQ), but not by that of ccDA (Fig. 8b).

Further, ccBA, but not ccDA, induced the expression of xenobiotic-metabolizing reporters: the phase I oxidative cytochrome P450 enzyme cyp-35B1 and the phase II conjugating enzyme gst-4 (Fig. 8c–f) involved in the detoxification of lipophilic compounds. The induction of cyp-35B1 was abolished by daf-16 RNAi, while that of gst-4 was abolished by skn-1 RNAi (Fig. 8c, d). These findings demonstrate that a specific stress and detoxification response involving a subset of DAF-16- and SKN-1-activated genes participate in the molecular defense against ccBA toxicity. In contrast, no apparent stress responses were detected upon ccDA exposure.

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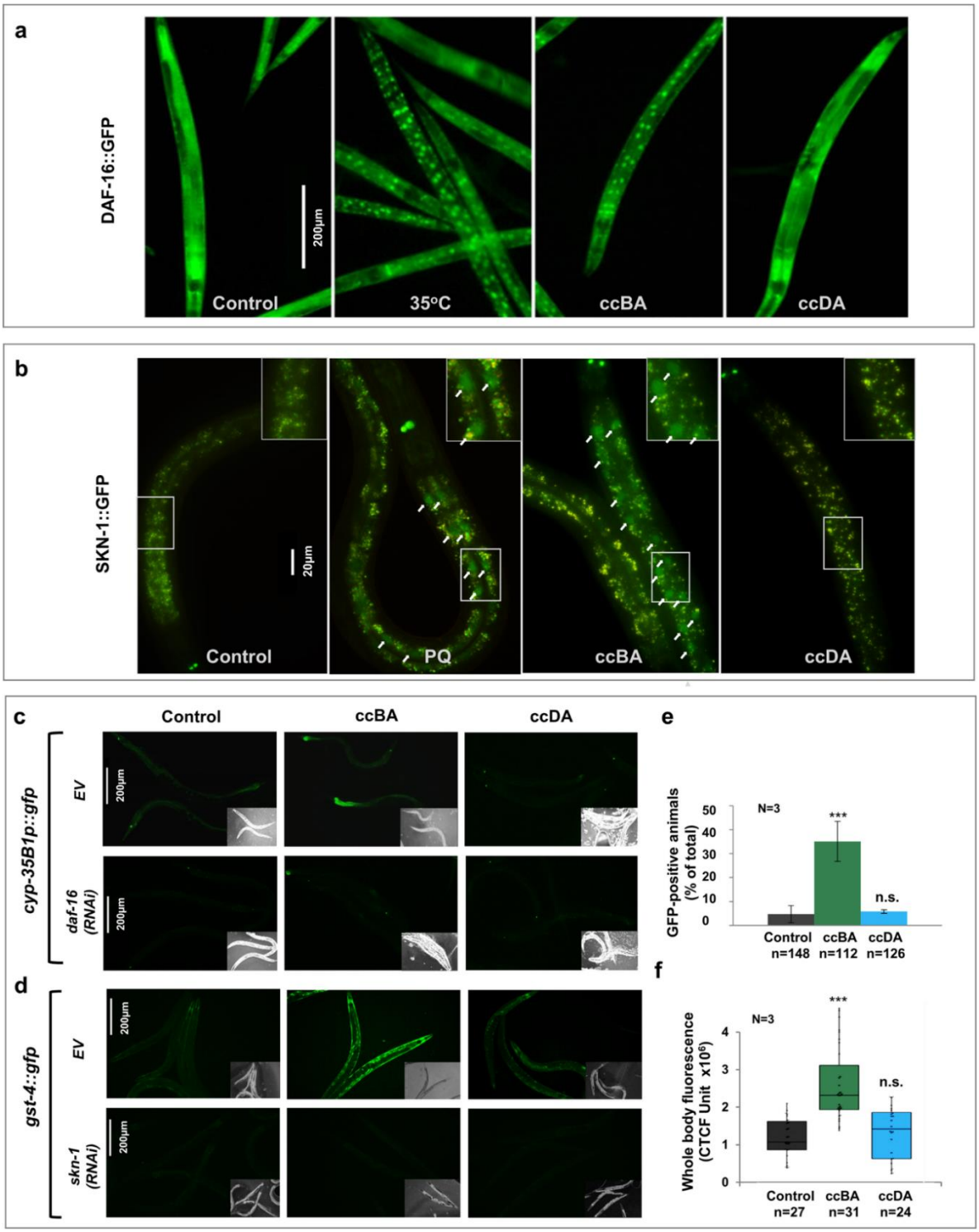


**Figure 8. ccBA, but not ccDA, activates specific systemic cytoprotective responses.** (a) Representative epifluorescent microscopic images of DAF-16::GFP nuclear translocation in response to a 50-min 35 °C heat stress (HS) or a 30-min exposure to 1 µl ccBA or 4 µl ccDA in young adults. (b) Representative epifluorescent microscopic images of SKN-1::GFP nuclear translocation in response to 4 mM paraquat (PQ) for 1 h, 1 µl ccBA or 4 µl ccDA for 30 min in L3 larvae. Please note the specific fluorescence of the larger nuclei labeled by arrows in the image and in the inset of some samples and the granular intestinal autofluorescence present in each sample. (c) Representative

epifluorescent microscopic images of cyp-35B1p::gfp expression in response to a 4-h exposure of 1 µl ccBA or 4 µl ccDA in worms fed by control empty vector (EV) and daf-16 RNAi. (d) Representative epifluorescent microscopic images of gst-4::gfp expression in response to a 4-h exposure of 1 µl ccBA or 4 µl ccDA in nematodes fed by EV and skn-1 RNAi. (e) Quantification of cyp-35B1p::gfp expression in response to a 4-h exposure to ccBA or ccDA in worms fed by control EV (top row of panel (c). (f) Quantification of gst-4::gfp expression in response to a 4-h exposure to ccBA or ccDA in nematodes fed by control EV (top row of panel d). Please note the lack of detectable fluorescent signal in nematodes fed by daf-16 or skn-1 RNAi. Data are expressed as mean ± SEM. N, number of independent experiments; n, number of animals. p values were obtained by one-way ANOVA with Fisher's LSD post hoc test (e) or unpaired Student's t test following the evaluation of normal distribution significance by the Kolmogorov-Smirnov test (f). The inter-experimental variation (%CV) for (f) was 20% (control), 22% (ccBA), and 25% (ccDA). n.s., not significant; ***$p < 0.001$

### 3.2.2 BA PC induced behavioral tolerance is dependent on efficient (cellular) stress and detoxification responses

The "fight-or-flight" response is an essential part of the general adaptation reaction to diverse stresses (Selye, 1973). Therefore, we asked whether the cytoprotective responses activated by ccBA which are known to induce physiological tolerance to various stresses might play a role in the generation of "fight-or-flight" (staying on or leaving the lawn) behavioral decisions. To this end, we preconditioned N2 and *daf-16* null mutant nematodes with ccBA and studied their food avoidance to ccBA. We found that naive *daf-16* mutants showed avoidant behavior comparable to wild-type; however, they failed to decrease their aversion in response to preconditioning (Fig. 9a). A similar phenotype was obtained by silencing the evolutionarily conserved molecular chaperone Hsp90, which was shown to regulate DAF-16 activity (Somogyvári, Gecse, & Sőti, 2018) (Fig. 9b). Likewise, *skn-1* silencing similarly prevented the development of behavioral tolerance, whereas the activation of SKN-1 by knocking down the WDR-23 protein responsible for its degradation (Choe, Przybysz, & Strange, 2009) augmented behavioral tolerance towards ccBA (Fig. 9c). Further, RNAi knockdown of daf-16 or skn-1 impaired survival to ccBA (Hajdú et al., 2021). In sharp contrast, after ccDA preconditioning, neither *skn-1* nor *wdr-23* RNAi altered the behavioral sensitization towards ccDA exposure (Fig. 9d). RNAi did not silence neuronal Hsp90 and SKN-1 isoforms (Papp et al., 2012; Somogyvári et al., 2018), in agreement with its inability to enter neurons (Winston, Molodowitch, & Hunter, 2002). These results demonstrate that specific cytoprotective responses induced by toxic ccBA exposure in non-neuronal cells confer physiological protection and actively participate in the development of behavioral

tolerance. Thus, the ability to mount stress-specific molecular "fight" responses downregulates the behavioral avoidance "flight" response.

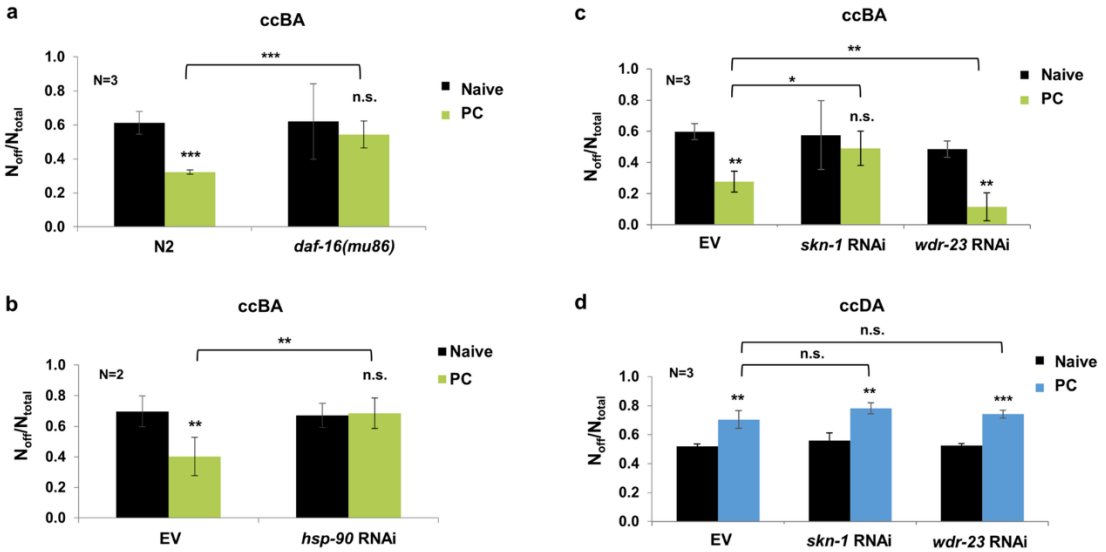


**Figure 9. Cytoprotection in non-neuronal cells contribute to behavioral tolerance in response to BA PC.** (a) ccBA-induced food aversion of naive and ccBA-preconditioned (PC) N2 wild-type and daf-16(mu86) mutant animals. (b) ccBA-induced food aversion of naive and ccBA-preconditioned nematodes fed by control empty vector (EV) and hsp-90 RNAi bacteria. (c) ccBA-induced food aversion of naive and ccBA-preconditioned nematodes fed by EV, skn-1, and wdr-23 RNAi, respectively. (d) ccDA-induced food aversion of naive and ccDA preconditioned nematodes fed by control EV, skn-1, and wdr-23 RNAi, respectively. Preconditioning and food leaving experiments were performed as indicated in Fig. 3. Data are expressed as mean ± SEM. N, number of independent experiments. p values were obtained by one-way ANOVA with Fisher's LSD post hoc test. n.s., not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

## 3.3 JNK-like MAP kinases and the NPR-1 neuropeptide Y receptor connect behavioral and physiological stress tolerance

The effect of extraneuronal and intracellular defenses in behavioral modulation upon stress suggested the involvement of inter-tissue signaling mechanisms. In *C. elegans*, neuroendocrine signaling is almost exclusively responsible for "top-down" inter-tissue communications, mainly via neurotransmitter release, the FMRFamide-type neuropeptide and the conserved stress-activated protein kinase (SAPK) pathways.

### 3.3.1 Stress-activated protein kinase (SAPK) pathways

The major downstream MAP kinases including the p38 ortholog PMK-1 as well as the JNK orthologs JNK-1 and KGB-1 guard physiological homeostasis in diverse stresses. Besides, a requirement of *kgb-1* in avoidance of toxic lawns and Toll-like receptor TIR-1

dependent inhibition of the *Pseudomonas aeruginosa* pathogen avoidance by *pmk-1* have been reported (Figure 8 and *58*, *59*).

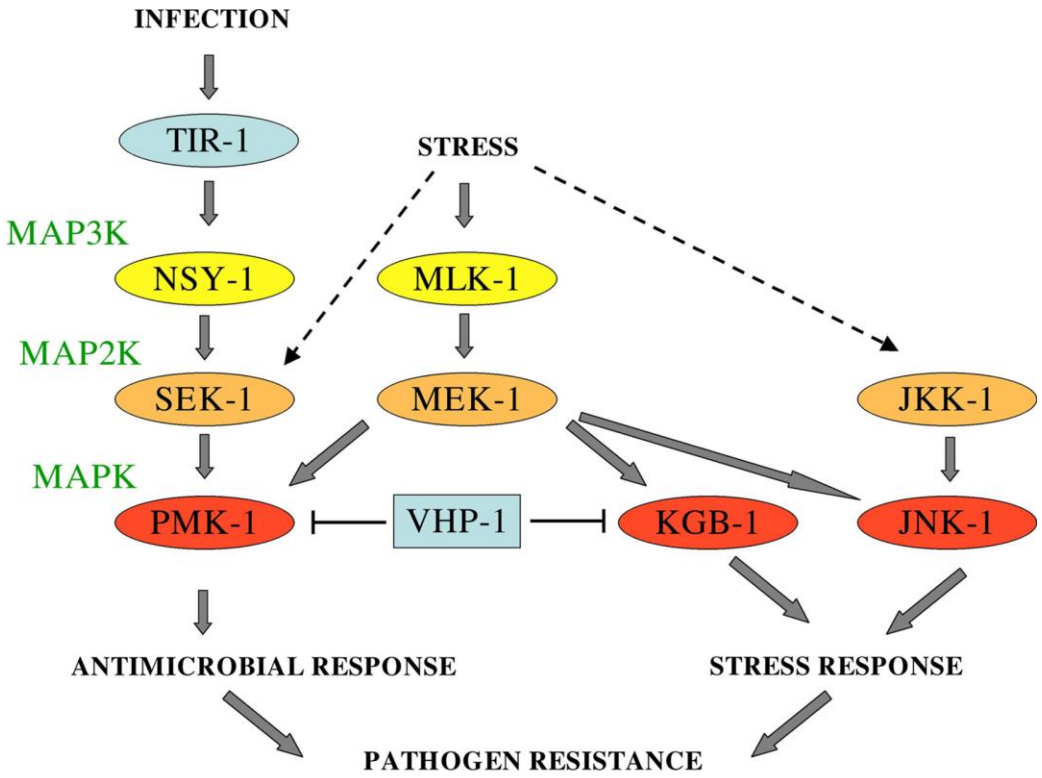


**Figure 10. SAPK signaling network in pathogen recognition and resistance. (from J. J. Ewbank, 2006).**

Hence, we tested the involvement of the respective mutants in ccBA aversion by subjecting naive and ccBA-preconditioned worms to the ccBA lawn leaving assay. Both *kgb-1* and *jnk-1* mutations diminished the aversion of naive worms to levels reminiscent of ccBA-preconditioned wild-type (Fig. 11a). *pmk-1* mutants rapidly and irreversibly paralyzed and died on the otherwise non-paralytic dose of ccBA; therefore, its role in ccBA avoidance could not be evaluated (Fig. 8a). Avoidance of ccDA also required, though to a smaller extent, *jnk-1* and perhaps *kgb-1*, which was at the threshold of significance, whereas *pmk-1* exerted no significant effect (Fig. 11b). These results suggest a role for JNK-like kinases in toxic odorant-elicited aversive behavior. SAPK members exert specific and overlapping roles in physiological defenses against various stresses. All three kinases help combat proteotoxic and heavy metal stress. Besides, PMK-1 promotes oxidative/xenobiotic, osmotic, and pathogen resistance partly via SKN-1. JNK-1 promotes heat stress resistance via DAF-16, while KGB-1 is required to protect from bacterial pore-forming toxins and ER stress. Hence, a parallel stimulation of behavioral aversion and physiological defenses by JNK-like kinases might be feasible.

Therefore, we exposed SAPK mutants to the lethal dose of the respective odors and tested their survival. Contrary to our assumption, *kgb-1* and *jnk-1* mutants, compared to wild-type, exhibited enhanced survival upon ccBA and unchanged survival upon ccDA exposure (Fig. 11c, d). These results are consistent with the lack of specific physiological defenses against ccDA, and a reciprocal effect of JNK-like kinases on ccBA-elicited responses: promotion of behavioral avoidance and attenuation of ccBA-specific physiological defenses (cf. Fig. 11a–d). As the ccBA concentration in the survival plates is uniform, the increased survival of *kgb-1* and *jnk-1* is independent of their reduced aversion. Therefore, either the JNK-like kinases separately promote aversion and suppress physiological stress response or the suppression of stress responses indirectly promotes aversion. Although our results do not allow a clear distinction, both alternatives confirm the reciprocal connection between physiological and behavioral defenses, observed with the cytoprotective regulators. Loss of *pmk-1* function did not significantly affect survival on ccDA (Fig. 11d), but completely hindered survival on ccBA (Fig. 11c), in agreement with the extensive paralysis observed on low-dose ccBA. Altogether, these findings suggest a physiological protection of vital importance conferred by *pmk-1* against ccBA toxicity, a requirement of JNK-like kinases to favor behavioral defense vs. ccBA-specific physiological defenses, and *jnk-1* (and *kgb*-1) to elicit avoidance as the sole available protective measure against ccDA.

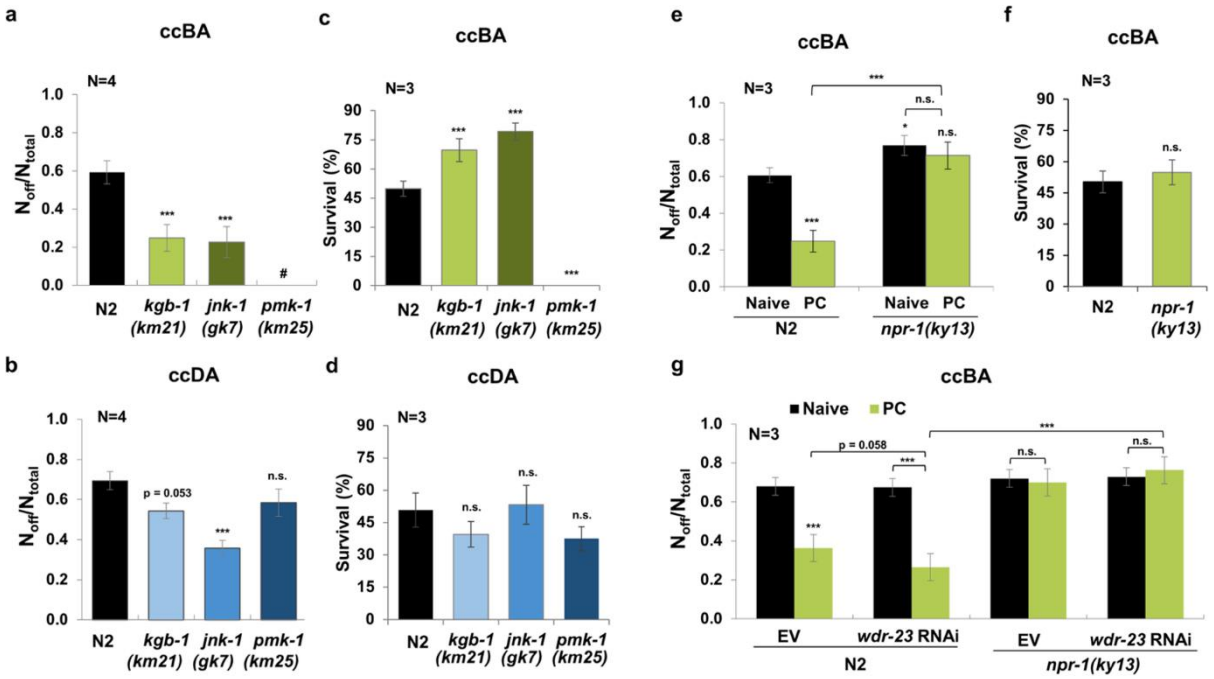


**Figure 11. SAPK-members and NPR-1 modulate behavioral and physiological tolerance in response to BA PC.** (a) ccBA-induced food aversion of wild-type and SAPK mutant worms. (b) ccDA-induced food aversion of wild-type and SAPK mutant worms. (c) Survival of wild-type and SAPK

mutant worms 14 h after a 3-h exposure to 8 µl ccBA. (d) Survival of wild-type and SAPK mutant worms 14 h after 3-h exposure to 16 µl ccDA. (e) ccBA-induced food aversion of naive and ccBA-preconditioned (1 µl for 4 h) N2 and npr-1 mutants. (f) Survival of N2 and npr-1 mutants 14 h after exposure to 8 µl ccBA for 3 h. (g) ccBA-induced food aversion of naive and ccBA-preconditioned (1 µl for 4 h) N2 and npr-1 mutants, fed by control empty vector (EV) or wdr-23 RNAi. Preconditioning and food leaving experiments were performed as indicated in Fig. 3. Data are expressed as mean ± SEM. N, number of independent experiments. p values were obtained by one-way ANOVA with Fisher's LSD post hoc test. n.s., not significant; ***$p < 0.001$

### 3.3.2 FMRFamide-type neuropeptide receptor signaling

The conserved neuropeptide Y receptor ortholog NPR-1 is an important integrator of various external and internal cues and modulates diverse physiological and behavioral responses including innate immunity, social vs. solitary feeding, arousal, and avoidance of P. aeruginosa.

We investigated the behavioral response of naive and ccBA-preconditioned *npr-1* mutants to ccBA in food leaving assays. *npr-1* mutants initially aggregated on the *E. coli* lawn, but in response to ccBA, they dispersed and left the lawn, similarly to wild-type animals. Strikingly, we observed a complete suppression of the behavioral tolerance in ccBA-preconditioned *npr-1* mutants (Fig. 10e). The increased aversive behavior of *npr-1* mutants could ensue from a compromised resistance to ccBA toxicity, as NPR-1 activates physiological defenses, such as PMK-1-dependent immunity in response to P. aeruginosa infection (Styer et al., 2008). However, the *npr-1* mutation did not affect survival upon lethal ccBA exposure (Fig. 11f), suggesting that wild-type NPR-1 does not engage physiological defenses, rather appears to integrate the internal signals of physiological homeostasis into the aversive response against ccBA. We tested this prediction by boosting SKN-1 activity in N2 and *npr-1* animals using *wdr-23* RNAi and subjecting them to the ccBA food leaving assay. Indeed, *wdr-23* RNAi improved the behavioral tolerance in preconditioned wild-type, but not in npr-1 nematodes (Fig. 11g), indicating the disconnection in physiological and behavioral defenses in the absence of *npr-1*. Due to the strong escape of ccBA-preconditoned npr-1 worms, which phenocopied the avoidance of ccDA lawns by ccDA-preconditioned wild-type and the lack of ccDA-induced physiological defenses, we did not study *npr-1* in ccDA conditions.

Altogether, these results suggest that SAPK-s and NPR-1 exert opposite effects and cooperate in fine-tuning physiological and behavioral "fight-or-flight" responses to protect homeostasis in toxic stress conditions.

### 3.4 Cytoprotective defenses during stress determine learned behavioral decisions to stress-associated olfactory cues

Previously we asked whether the prior experience of odor toxicity and the different efficiency of physiological defenses influence nematodes to make optimal choices upon encountering the olfactory cues present at the time of stress. To examine this, my colleague Eszter Gecse investigated alterations in behaviors towards attractive (1%) doses of DA and BA after preconditioning with toxic, undiluted doses of the respective odors. She found the generation of distinct, avoidant, or tolerant learned behaviors to stress-associated olfactory cues of ccDA or ccBA, respectively (see Fig. 9 in (Hajdú et al., 2021)).

The elicitation of learned stress-reactive behaviors by olfactory cues raises the possibility that the learned experiences form distinct memories to cope with anticipated future insults. On the other hand, forgetting irrelevant, non-recurrent experiences is also important as both the organism and the environment are changing. We tested the stability of newly acquired behaviors by subjecting worms to ccBA lawn leaving assays immediately or 2 h after preconditioning with ccBA. We observed that a 2h recovery period after a single ccBA preconditioning for 2 or 4h significantly attenuated behavioral tolerance against ccBA in the food leaving assay (Fig. 12a). The increased lawn avoidance after recovery might either be due to the decrease in stress-induced physiological defenses or in the loss of the new, yet unstable changes in neural representation, forgetting. Repeated training sessions with inter-trial “rest” intervals, called spaced training, potently amplifies learning efficiency via memory consolidation (Smolen et al., 2016). Spaced training is known to induce stable memories over 2h in *C. elegans* (Kauffman, Ashraf, orces-Zimmerman, Landis, & Murphy, 2010). Hence, we tested whether spaced training, by counteracting forgetting, might increase the persistence of the acquired behavioral tolerance to ccBA after the recovery. The induction of aversive memory is optimal beyond three training sessions with 10min “rest” intervals (Amano & Maruyama, 2011). Therefore, we employed a protocol of spaced training using four times 1-h exposures to 2 µl ccBA on large food lawns with 10-min rest times during the washing steps in between (Fig. 12b). Then, half of the nematodes were subjected to ccBA lawn leaving assays immediately after training, the other half after a 2-h recovery period. We found that immediately after pre-exposures, both the single preconditioning and the spaced training resulted in a similar suppression of ccBA avoidance, suggesting similar levels of behavioral tolerance elicited by both protocols (Fig. 12a, c). However, the behavioral tolerance was entirely retained after a 2-h recovery in spaced-trained nematodes (Fig. 12c). We also examined

whether repetitive encounters with the same dose of ccDA as in single preconditioning might influence food avoidance behavior in the presence of 1%, innately attractive DA. We observed that spaced-trained worms exhibited robustly increased food leaving behavior against 1% DA (Fig. 12d), compared to that elicited by a single 4-h preconditioning (see Fig. 7c, "DA PC" column), reaching a similar aversion index to that elicited by ccDA (see Fig. 7c, "ccDA Naive" column). Moreover, the extent of the avoidant behavior was entirely preserved after the 2-h recovery (Fig. 12d). It appears that after the ccDA exposure worms changed their preference towards the non-toxic 1% DA olfactory cue. Thus, spaced training with ccBA or ccDA leads to the stabilization of respective stress-associated memories over 2 h, which upon retrieval give rise to either tolerant, coping "fight," or avoidant "flight" behavioral responses.

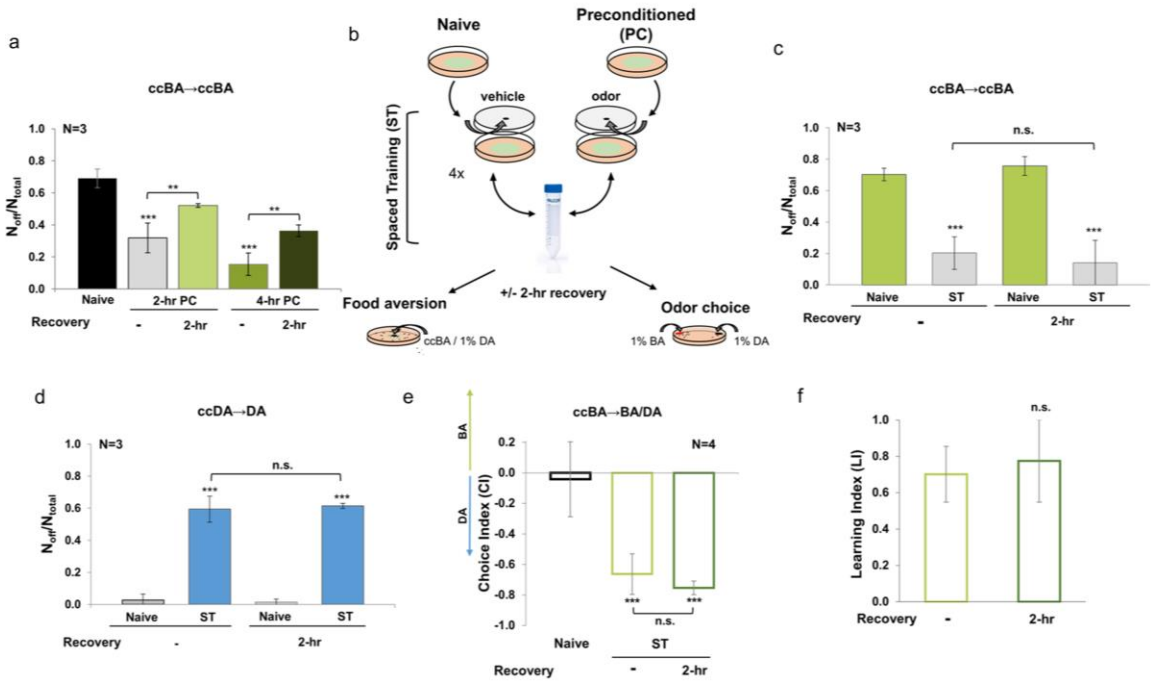


**Figure 12. Efficient or deficient cytoprotection elicited behaviors are preserved in distinct coping or avoidant memories.** (a) Effect of a 2-h recovery period on ccBA-induced food aversion elicited by ccBA preconditioning using single 2-h (2-h PC) or 4-h (4-h PC) exposures. (b) Experimental design for spaced training (ST) using toxic odors. Animals were exposed to a hanging drop of undiluted odor (2 µl ccBA or 4 µl ccDA, preconditioned, PC) or vehicle (naive) using a 4 × 1-h spaced training protocol with 10-min inter-trial "rest" times during the washing step. Animals were assayed for food aversion or odor preference immediately or after a 2-h recovery period. (c) Effect of a 2-h recovery period on ccBA-induced food aversion elicited by ccBA spaced training. (d) Effect of a 2-h recovery period on lawn avoidance in the presence of 1% DA elicited by ccDA spaced training. (e) Effect of a 2-h recovery period on odor choice between 1% BA and DA elicited by ccBA spaced training. Choice

indices were calculated as CI = (# on BA − # on DA)/(# on BA + # on DA). (f) Learning indices from (e), calculated as LI = CI (naive) − CI (preconditioned). Error bars represent mean ± SEM. N, number of independent experiments. p values were obtained by one-way ANOVA (for food leaving assays) and by two-way ANOVA (for odor choice assays) with Fisher's LSD post hoc test.t. n.s., not significant; **$p < 0.01$, ***$p < 0.001$

Finally, we asked how the coping memory affects the choice between the stress-associated and a natural attractive odor olfactory cue. Spaced training with ccBA almost entirely shifted the preference towards DA (Fig. 12e), potentiating the change already observed by the single preconditioning (see Fig. 12g, h). Moreover, the robustly shifted odor preference evoked by spaced training was retained after a 2-h recovery (Fig. 12e) resulting in stable storage and retrieval of the acquired memory (Fig. 12f). The stability of memories generated by spaced training is consistent with the literature data (Amano & Maruyama, 2011). Hence, reinforcement of learning by spaced training led to the augmentation and stabilization of the acquired behavioral changes induced by ccBA and ccDA.

The complete shift of preference from BA to DA shows an apparent similarity to the complete shift of preference from DA to BA after the single preconditioning with ccDA (Gecse et al., 2019). Nonetheless, in contrast to the compelling avoidant "flight" behavior to the memory of uncompensated physiological harm, the memory of physiological protection not only provides the ability to cope with real or anticipated toxicity for food, but also allows a flexible decision to spare resources when the organism also perceives the olfactory cue of a potentially toxin-free food. This result also suggests that the memory of a stressful insult contains the representation of the original valence of the olfactory cue, the internal experience of stress-induced harm, and the activated physiological protection. Taken together, learned behaviors originating from adequate or inadequate physiological responses to stress generate acquisition of distinct coping or avoidant memories.

## 3.5 Cellular defense memories to cope with anticipated stress

Worms can encounter their natural pathogens or pathogen associated toxins throughout life. Recognizing harmful stimuli prior to direct tissue damage is the most important to preserve organismal integrity and homeostasis of core cellular processes. Some pathogens, such as the Gram-negative *Pseudomonas aeruginosa* secretes naturally attractive odorants developed during successful co-evolution, resulting in the typical phenomenon of initial attraction and delayed aversion of worms, a learned aversive behavior similar to that seen in case of benzotaxis assays (Nuttley et al., 2001; Zhang, Lu, & Bargmann, 2005). The hypothesis that

cytoprotective memories would be retrievable by conditioned cues gained support from the findings of Alon Zaslaver's (Eliezer et al., 2019) and our groups (Gecse et al., 2019). On the one hand, learned cytoprotective defenses might contribute to influence decision making and might confer a successful defense strategy for *C. elegans* against impending adverse events.

### 3.5.1 Memory retrieval of toxic volatile stress induces DAF-16/FOXO nuclear translocation

We discovered that ccBA preconditioning (BA PC) enforces an efficient physiological fight response to imminent danger, influencing decision making of subsequent associated cues. We hypothesized that the coping behavioral memory might be accompanied by the formation and retrieval of physiological stress defense memory by the CS (olfactory cue). For this reason, we carried out spaced training with stress-inducing ccBA (BA ST) and monitored DAF-16 translocation upon re-exposure to conditioned, 1% odor cues (Fig. 13a). Interestingly, approximately two-fold elevation of nuclear DAF-16 was observed in animals exposed to 1% BA after BA ST, compared to naïve conditions (Fig. 13a, b). These results indicate that retrieval of toxic stress memory by associated sensory cues re-activates a stress-specific regulator to ensure efficient cellular protection. However, the confirmation of a neural process as well as its physiological relevance requires further investigations.

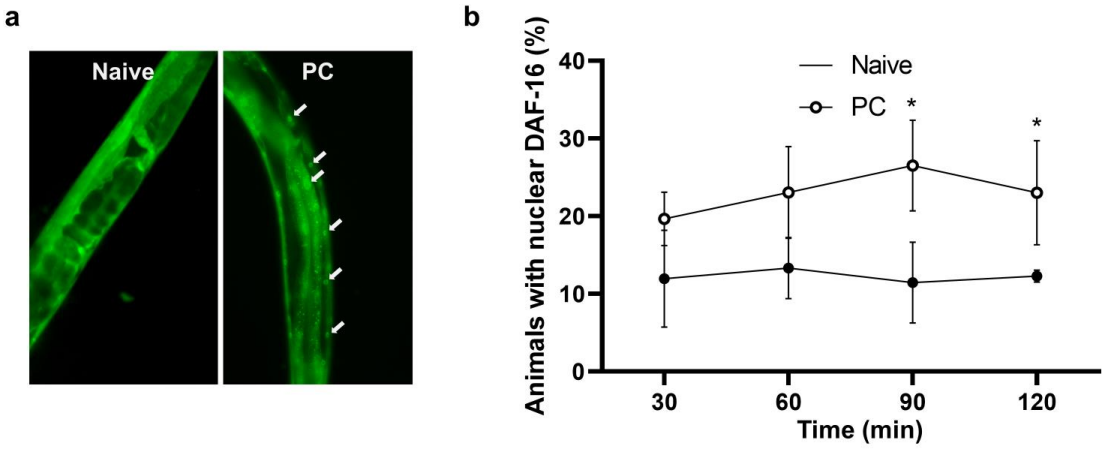


**Figure 13. Spaced training of BA PC generates cytoprotective memory of DAF-16 translocation.** (a) Representative epifluorescence microscopy images of Naive and BA ST animals after 30 minutes exposure of 1% BA. Arrows indicate nuclear DAF-16::GFP. (b) Time curves of naive and PC animals exhibiting DAF-16 nuclear localization upon 1% BA exposure. N = 2 the number of independent assays with at least 20 animals per condition. p values were generated by two-way ANOVA followed by Fisher's LSD post-hoc test. n.s, not significant, *$p < 0.05$

### 3.5.2 Retrieval of imprinted cytoprotective memory does not appear to enhance adult stress tolerance

Imprinting is a special form of associative memory acquired during a specific time window of early age, the L1 larval stage in *C. elegans*. In strict relation to this study, my colleague Eszter Gecse discovered that the L1 larval stage memory of toxin-induced expression of cytoprotective reporters can be retrieved by toxin associated *E: coli* OP50 olfactory cues in adults (Gecse et al., 2019). Consistently, we hypothesized that a stress inducing dose of paraquat (PQ PC) or antimycin (AM PC) treatment during the time window of imprinting might affect not only physiological AM or PQ tolerance of adults, but the memory retrieval of stress by pre-exposure to associated olfactory cues might enhance stress tolerance. To address these questions, adult worms exposed to AM or PQ on *E. coli* OP50 bacteria in the L1 larval stage for 24 hours, washed and grown on toxin-free *Bacillus subtilis* lawn to adulthood. Then, adults were placed onto plates containing BS or OP50 lawn six hours prior to a lethal toxic stress using the same toxins as in the L1 stage (Fig. 14a). Toxin exposure during the L1 stage induced an approximately twofold increase in survival of adults compared to naive animals (Fig. 14b, c). However, the re-encounter with the OP50 sensory cues before lethal stress neither altered survival rates in naive nor in toxin-imprinted worms (Fig. 14b, c). Thus, early life toxin exposure at the doses employed is hormetic and induces a lasting and robust stress tolerance in adulthood, which is not further enhanced by retrieval of the imprinted memory. Perhaps toxin-induced tolerance at early age is so robust that further elevation by neural imprinting is not detectable at these toxin doses. We therefore conclude the necessity of a more precise time- and dose-dependent measurement of physiological tolerance following L1 toxin treatment.

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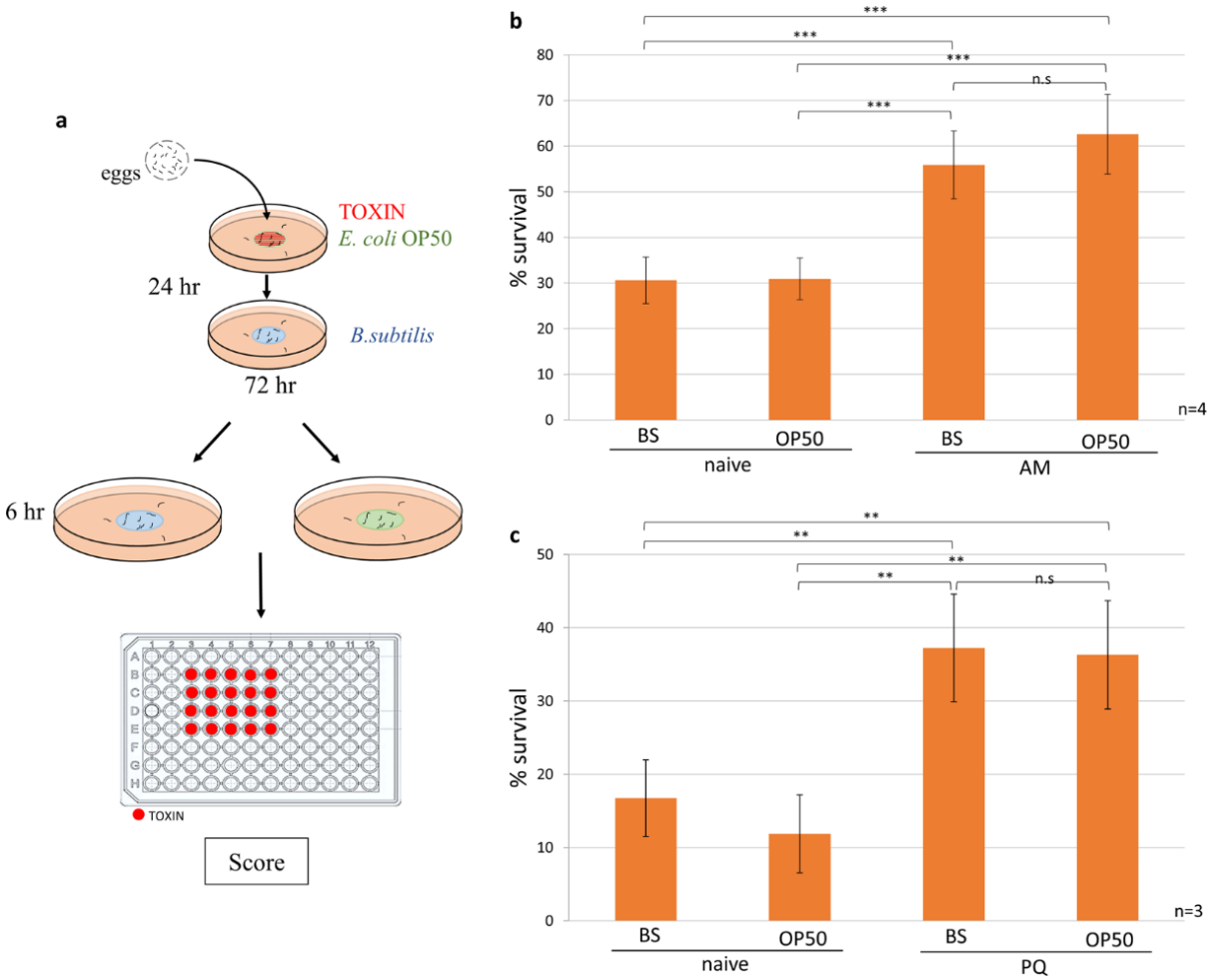


**Figure 14. Imprinted early-life toxin stress confer adult physiological tolerance independently of food bacteria.** (a) Schematic of the adult toxin tolerance assay. Effect of early life AM (b) or PQ (c) exposure and the re-encounter with toxin-associated OP50 cues on the survival rates of adult worms subjected to the same toxins. n = number of independent assays. p values were generated by ANOVA followed by Fisher's HSD post-hoc test. n.s, not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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# 4 Discussion

In the concept of stress biology, adequate response to environmental alterations is essential to preserve organismal homeostasis. During my experimental PhD work, we have established a paradigm of volatile toxicity to measure adequate or inadequate responses and learning processes towards hormetic stress or distress, respectively. Specifically, we developed an experimentally straightforward technique to investigate the link between adaptive cellular and behavioral responses in time of toxic stress. We have demonstrated that the attractant benzaldehyde (BA) and diacetyl (DA) in undiluted form induce toxicity and distinct strategies of protection when employed as preconditioning exposure. In addition, BA PC elicited cellular stress responses to induce physiological and behavioral tolerance, while distress caused by DA PC triggered elevated and sustained aversion. Hence, our paradigm established a cytoprotective stress model, in which evolutionary conserved cellular stress and detoxification signaling pathways give rise to a molecular "fight" response. We also revealed the role of inter-tissue signals that linked cytoprotection and behavior. Furthermore, associative memory and behavioral decisions following stressful experience of (spaced training) by BA or DA, respectively, were influenced by the efficacy of cellular protection. Our findings also suggest that the stress-induced cytoprotective response might form a physiological defense memory which might prepare the organism for anticipated future stresses (Figure 15).

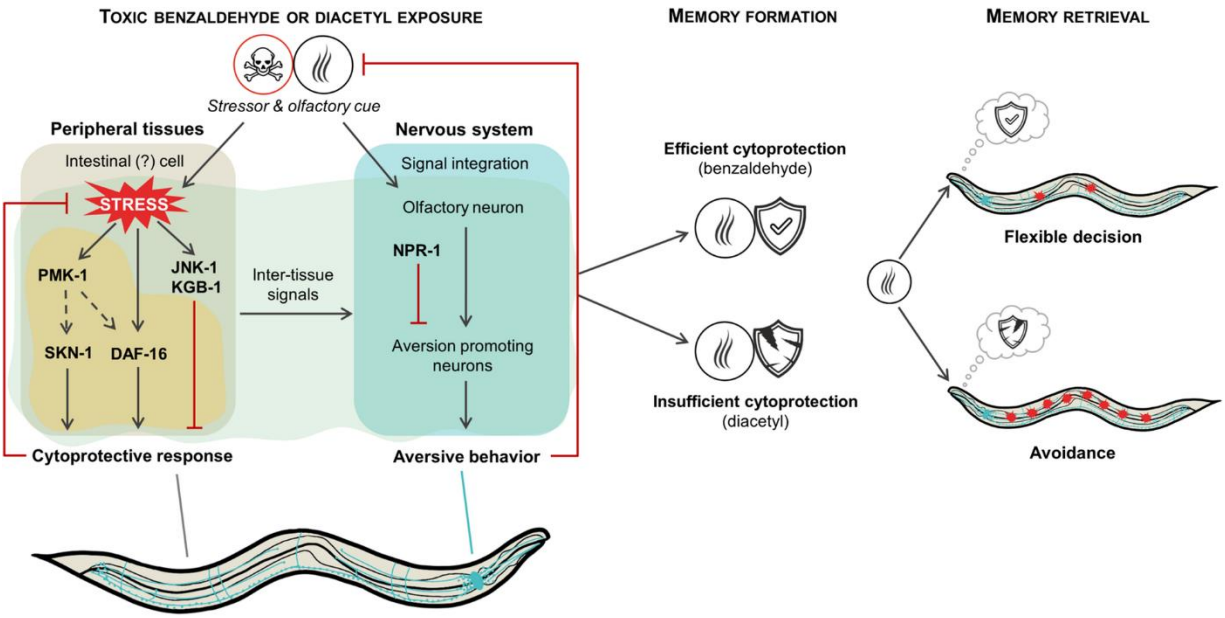


**Figure 15. Model for the regulation of learned behavioral decisions by cytoprotective responses.** Undiluted odorants induce stress in non-neuronal cells. Cells emit inter-tissue danger signals to the nervous system which require JNK-like kinases and are integrated with other signals to control aversion.

(The site of action of KGB-1 and JNK-1, although indicated in the peripheral cell, is yet undefined.) Benzaldehyde-specific cytoprotective responses (beige area) alleviate stress and danger signals, which diminish aversion via the neuropeptide receptor NPR-1. Reinforcement of these experiences forms a memory of protection, which upon retrieval by the olfactory cue allows a flexible decision depending on the external context, such as the availability of other, stress-free food sources. Insufficient cytoprotection (diacetyl) aggravates stress which leads to behavioral sensitization and forms a memory of danger, which upon retrieval compels to avoidance. Dashed lines denote results inferred from other studies, see the "Discussion" section for details

## 4.1 Stress-induced cytoprotective responses and learned behavioral decisions

It is well defined that repellent-induced avoidance is generated by the ASH, AWB or ADL chemosensory neurons. Sensory integration of these inputs is mostly responsible for aversive associative learning and non-associative context-conditioning of toxic stimuli (Amano & Maruyama, 2011; Ardiel & Rankin, 2010; Sasakura & Mori, 2013). However, the reason for the concentration dependent aversion of innately attractive odors such as BA and DA, remained unclear. We investigated this phenomenon in the presence of food bacteria, where multiple sensory inputs guide animals' behavior. The observed odorant induced avoidance excludes the possibility of habituation or olfactory adaptation, and clearly indicates aversive behavior. The fact that (i) the previously monitored chemotactic behavior was largely altered by starvation (Colbert & Bargmann, 1995; Nuttley et al., 2001; Pereira & van der Kooy, 2012), and our findings on (ii) the progressive dose-dependent paralysis and compromised thermotolerance, (iii) activation of specific cell-autonomous stress responses of DAF-16/FOXO and SKN-1/Nrf-2 by ccBA and (iv) the phenomenon of *daf-16, skn-1* and *hsp-90* dependent behavior together led us to the conclusion that odorant induced lawn avoidance was not only influenced by chemosensory input, but also by inputs form the surveillance of cellular homeostasis. Indeed, both ccBA and ccDA were found to promote loss of tissue integrity and death. We also observed similar aversive behavior and toxicity using undiluted methyl salicylate which possesses a highly similar aromatic structure to BA (Fig. 7 of (Hajdú et al., 2021). The induction of apparently identical cellular stress responses upon benzaldehyde and methyl salicylate (see Figure 5 of (Hajdú et al., 2021)) suggests structure-specific recognition by detoxification machinery. On the other hand, ccDA induced sustained food avoidance exhibited the characteristics of distress caused by uncompensated toxicity – which was confirmed by the increased lethality upon ccDA as well as by lack of apparent cytoprotection.

During investigations of DAF-16::GFP localization by fluorescence microscopy in

response to ccBA, we detected an interesting phenomenon: wild-type animals showed a rapid induction of intestinal autofluorescence (Hajdú G., Sőti C., unpublished observations). We found that the elevation of autofluorescence was localized to intracellular gut granules of enterocytes, the only existing class of *C. elegans* Lysosome-related Organelles (LROs). An interesting question would be whether LRO function might contribute to physiological and behavioral tolerance of preconditioned animals. LROs in *C. elegans* were reported to accumulate autofluorescent material with age, which appears a suitable marker of healthspan (Gerstbrein, Stamatas, Kollias, & Driscoll, 2005). The discovery that specific toxin mediated stress stimulates a yet unexplored rapid elevation of LRO storage gives rise to the assumption that LROs might participate in systemic stress and detoxification processes, which remains to be experimentally tested.

Beyond cellular adaptive responses, we demonstrated not only elevated physiological tolerance but reduction of food avoidance in response to a ccBA pre-exposure (preconditioning, PC), which we named behavioral tolerance. Moreover, the effect of BA PC was dependent on efficient cytoprotection as disruption of all DAF-16, SKN-1 and HSP-90, respectively, compromised behavioral tolerance. RNA interference in *C. elegans* is restricted to extraneural tissues (Timmons, 2006), therefore alterations in BA PC induced behavioral tolerance in animals that fed by *hsp-90, skn-1* or *wdr-23* RNAi is highly likely to result from neuroendocrine signals. The hormetic effects of preconditioning induced stress or cross-tolerance *via* the activation of key molecular stress pathways have been characterized. For instance, preconditioning with mild heat stress resulted in increased survival on lethal heat or pathogen exposure in an *hsf-1* dependent manner (Hsu, Murphy, & Kenyon, 2003; V. Singh & Aballay, 2006). In our experimental model, we demonstrated that hormesis of BA PC elevates not only physiological and behavioral tolerance towards toxic benzaldehyde, but also towards methyl salicylate. Furthermore, our volatile toxicity model allows the investigation of learning and memory formation in response to BA PC induced cytoprotection.

Inter-tissue signals between neuronal and non-neuronal cells are evolutionary fundamental elements of cellular communication. In *C. elegans*, all the ancient TGF-β, SAPK pathway as well as FMRFamide system oversees numerous cellular processes of self-protection and play key role in cell-nonautonomous “top-down” (i.e. neuron-to-periphery) regulation by the nervous system (Andrusiak & Jin, 2016; Foster et al., 2020; J. Singh & Aballay, 2020; Soto, Goetting, & Van Buskirk, 2019; Styer et al., 2008). However, especially in recent reports of *P. aeruginosa* pathogenesis, the direction of danger signal is reversed in

response to perception of intestinal bloating by NPR-1, ROS generated in enterocytes sensed by ASER SOD-1, disruption of core cellular processes perceived by p38 MAPK, DAF-7/TGF-β as well as serotonergic and FMRFamide system to alter behavior (Horspool & Chang, 2017; Kim et al., 2002; Lee & Mylonakis, 2017; Melo & Ruvkun, 2012; J. Singh & Aballay, 2019, 2020). Our results confirmed that two members of SAPK signaling, *kgb-1* and *jnk-1* promote ccBA induced food avoidance, furthermore *pmk-1* is required for BA PC induced physiological, while *npr-1* is necessary for behavioral tolerance.

SAPK members and *pmk-1* were characterized to act in pathogen invasion and oxidative stress via regulation of DAF-16, ATF-7 and SKN-1 (Ewbank, 2006; Fletcher et al., 2019; Inoue et al., 2005). In contrast to the moderate lethality of *skn-1* RNAi treated animals upon ccBA, the exceptionally strong effect of PMK-1 on survival suggests both SKN-1 dependent and independent routes of stress regulation. For this reason, the intriguing next experimental step would be a widespread epistatic analysis. Remarkably, other studies further confirm that KGB-1 like kinases and NPR-1 mediates microbial aversion (Lee & Mylonakis, 2017; Melo & Ruvkun, 2012), whereas our results indicating elevated tolerance of *kgb-1* and *jnk-1* animals suggest reciprocal regulation of behavioral and physiological defenses. Interestingly, the reduced food avoidance of physiologically BA-resistant *wdr-23 RNAi* nematodes was not manifested in *npr-1* mutants, demonstrating epistatic role of inter-tissue signaling over the cellular stress pathway. Contrary to the role in sensation of intestinal bloating, *npr-1* seems to suppress activation of behavioral avoidance program in response to ccBA toxicity. Nevertheless, contrasting roles of *npr-1* was also observed in case of pathogen-specific immune defenses (Nakad et al., 2016). However, minor requirement of SAPK members in ccDA induced lawn avoidance suggest toxin specific action of these pathways to regulate behavior. Consequently, along with previous observations, our results using a simple experimental paradigm further confirmed the existence of a “bottom-up” direction of communication driven by evolutionary ancient molecular machineries.

We confirmed that toxicity of undiluted odorants and the respective, efficient or deficient cytoprotective responses form distinct, coping or avoidant associative memories to olfactory cues. Flexible decision making upon 1% odors after BA PC and BA ST is an evolutionary successful strategy for the animal during changing environment to anticipate insults and coordinate cellular protection and behavior. Coping with stressful memories is extremely important in case of encountering food sources harboring olfactory cues experienced during a prior stress, when the organism is required to make priorities whether it is rewarding or not to

invest into a “fight” response rather than choosing the avoidant “flight” response.

Our findings on diacetyl-induced avoidant associative memory strongly suggest a causative role of deficient physiological defenses during stress. Further experiments on the behavior of worms with genetically disrupted cytoprotective mechanisms might confirm the link between the physiological defenses and behavior. Then, identification of neurons and mechanisms involved in the signaling might shed light on the neuronal perception of internal milieu and cellular homeostasis. Manipulation of these pathways might help extinguish the maladaptive memories, such as avoidant behaviors, when physiological defenses work well, or when there is an accidental association between a stress and a sensory cue.

## 4.2 Stress response evoked by a stress associated olfactory cue: a cytoprotective memory

The largely unexplored concept of cytoprotective memory defines the re-activation of cellular stress pathways upon sensory retrieval of stressful memories. It was shown in the case of induction of stress and detoxification reporters by imprinted sensory cues associated with toxic stress ((Gecse et al., 2019) our study), activation of transcriptional DAF-16 response by associative memory of isoamyl alcohol (CS) and starvation (US) (Eliezer et al., 2019) as well as our observation on DAF-16 nuclear translocation upon 1% BA also suggests a chemosensory retrieval of BA-elicited toxic stress memory. Since 1% BA-induced DAF-16 activation occurs only in worms preconditioned by undiluted BA, it certainly involves a neural process. Our findings gain support from those of Zaslaver et al. demonstrating a learning process involving the AWC neurons and serotonin in the re-activation of DAF-16 by the starvation-associated olfactory cue (Eliezer et al., 2019). However, further experiments are required to clarify the mechanisms involved in our observation and its physiological significance.

## 4.3 Retrieval of an imprinted cytoprotective memory fails to enhance stress tolerance

We have observed elevated physiological tolerance by early life toxic AM and PQ treatment. Moreover, this hormetic effect was not further elevated in response to stress-associated olfactory cues. This finding suggest that perhaps imprinted physiological memories might not enhance adult stress tolerance. Alternatively, the strength of the preconditioning-induced, possibly epigenetic cytoprotective defense is so intense that any further difference by memory retrieval cannot be visible in our experimental conditions. Further experiments are required to distinguish between these possibilities. Yet, the observation on the retrieval of

learned adult physiological responses in adulthood suggests that the persistent elevation of such responses might result in long-term depletion of anabolic processes by constant alertness of cytoprotective machineries. In this context, our results demand further experiments to discover long term (negative) physiological and behavioral effect of early life stresses.

## 4.4 Closing remarks, ideas

In summary, our findings show an intricate connection between physiological and behavioral defenses as well as learned responses in anticipated stress. As physiological defenses are conserved, such connection between them and learned behavior might operate in higher organisms. Furthermore, the idea that past (i. e. adult learned or imprinted) experiences of cellular stress might shape future decisions and physiological processes in response to sensory stimuli would provide a plausible mechanism to explain complex behavioral and somatic symptoms of various human mental, functional neurological and psychosomatic disorders such as phobias, eating disorders, PTSD or irritable bowel disease. Whether inefficient and/or learned physiological defenses play a role in the formation of learned exaggerated avoidance and perceived stress, is a relevant question of future studies. For that, simple animal models, such as *C. elegans* might offer a tractable tool to identify the evolutionary conserved roots of memory formation and potential therapeutic targets at the molecular level to extinguish maladaptive memories.

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# 5 Conclusion

The aim of my PhD work was to deepen our knowledge about the impact of cytoprotective “fight” response (of non-neuronal cells) to memory formation and retrieval in time of future stress.

Our novel findings on the role of cytoprotective responses in adult and early life stress models are the following:

- We have established a nematode model of volatile-induced toxic stress:
- We characterized the impact of stress-induced cytoprotective responses on memories to cope with future anticipated stress:
    1. Toxic benzaldehyde, but not diacetyl induces activation of evolutionary conserved major stress regulators.
    2. Toxic benzaldehyde preconditioning forms a memory of behavioral tolerance through the activation of specific cytoprotective responses.
    3. Lack of apparent cytoprotection by toxic diacetyl enhances behavioral sensitization and associative aversive memory.
- We found the nuclear translocation of DAF-16 in non-neuronal cells evoked by the stress-associated olfactory cue.
- We found that early life toxic stress by antimycin A or paraquat induced adult stress tolerance, which was not further enhanced by stress-associated olfactory cues.

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# 6 Summary

In response to environmental adversities, organisms evolved “fight or flight” strategies to overcome or elude danger. Sensory perception of harmful stimuli elicits behavioral aversion, whereas disturbance in physiological homeostasis generates systemic stress, detoxification and immune responses to eliminate noxious agents and maintain cellular homeostasis. It is known that physiological stress influences both behavior and physiological tolerance to future stresses. However, their relationship is largely unknown.

The nematode *C. elegans* is a widely used model organism in stress biology and neuroscience due to its transparency, evolutionarily conserved cellular stress and detoxification responses, mapped connectome as well as its easy-to-monitor behavioral patterns, learning and memory formation. The goal of my doctoral project was to investigate the link between adaptive cellular and behavioral responses, as well as to deepen our understandings about the influence of toxic stress on associative behavioral and cytoprotective memories.

First, we established a nematode model of toxic volatile stress using undiluted food-derived odorants benzaldehyde (BA) and diacetyl (DA). We described that exposure of *C. elegans* to BA and DA induced toxicity and aversive behavior. BA preconditioning activated systemic cytoprotective responses involving DAF-16/FOXO, SKN-1/Nrf and Hsp90 in non-neuronal cells, which conferred both physiological (increased survival) and behavioral tolerance (reduced food avoidance) to BA exposure. In contrast, DA preconditioning augmented avoidance, weakened physiological DA tolerance and did not induce apparent molecular defenses. The inter-tissue connection between cellular and behavioral defenses was mediated by JNK-like stress-activated protein kinases and the neuropeptide Y receptor NPR-1. Retrieval of spaced training induced memory led to avoidance of food contaminated by diluted DA and context-dependent behavioral decision to avoid BA only if there was an alternative, food-indicative odor. Moreover, re-encountering the olfactory BA cue induced the nuclear translocation of DAF-16::GFP in BA trained nematodes, which suggested the formation of a physiological defense memory. An imprinted physiological defense memory was reported earlier by our group in response to early life exposure to antimycin A or paraquat. Now, we found that both toxic stresses elevated physiological toxin tolerance in adulthood, which was not further influenced by the olfactory retrieval of the imprinted cytoprotective memory.

In summary, my doctoral work reveals a regulatory link between cellular adaptive responses and learned behavior as well as suggest the formation of a physiological defense memory, which may facilitate self-protection in anticipated stresses.

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# 8 Bibliography of the candidate's publication

### Publications related to this thesis

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